PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

COMMUNICATION OF
INTERNATIONAL APPLICATIONS

(PCT Article 20)

To:

United States Patent and Trademark
Office
(Box PCT)
Washington D.C. 20231
United States of America

in its capacity as designated Office

Date of mailing:
02 May 1996 (02.05.96)

The International Bureau transmits herewith copies of the international applications having the following international application numbers and international publication numbers:

International application no.:	International publication no.:
PCT/GB95/01791	WO96/03997

CORRECTED VERSION
VERSION CORRIGEE

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer:
J. Zahra
Telephone No.: (41-22) 730.91.11

Copy for the Elected Office (EO/US)

PCT/GB95/01791

PATENT COOPERATION TREATY

1806

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

McCALLUM, William, Potter
Cruikshank & Fairweather
19 Royal Exchange Square
Glasgow G1 3AE
ROYAUME-UNI

RECEIVED
JUN 17 1997
GROUP 1800

Date of mailing (day/month/year) 29 January 1997 (29.01.97)	
Applicant's or agent's file reference	**IMPORTANT NOTIFICATION**
International application No. PCT/GB95/01791	**International filing date** (day/month/year) 28 July 1995 (28.07.95)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
THE UNIVERSITY COURT OF THE UNIVERSITY OF GLASGOW No. 2 The Square University Avenue Glasgow G12 8QQ GB	GB	GB
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address	State of Nationality	State of Residence
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

3. Further observations, if necessary:
The above-named applicant has been deleted from the record.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Ann Bardini
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 730.91.11

Form PCT/IB/306 (March 1994)

001376730

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Washington D.C. 20231
United States of America

in its capacity as elected Office

Date of mailing (day/month/year) 20 March 1996 (20.03.96)	
International application No. PCT/GB95/01791	**Applicant's or agent's file reference**
International filing date (day/month/year) 28 July 1995 (28.07.95)	**Priority date** (day/month/year) 29 July 1994 (29.07.94)
Applicant MACLEAN, Alasdair, Roderick et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

27 February 1996 (27.02.96)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

M. Abidine

Telephone No.: (41-22) 730.91.11

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

McCALLUM, William, Potter
Cruikshank & Fairweather
19 Royal Exchange Square
Glasgow G1 3AE
ROYAUME-UNI

Date of mailing (day/month/year)
30 October 1996 (30.10.96)

Applicant's or agent's file reference

IMPORTANT NOTIFICATION

International application No.
PCT/GB95/01791

International filing date (day/month/year)
28 July 1995 (28.07.95)

1. The following indications appeared on record concerning:

☐ the applicant ☐ the inventor ☐ the agent ☐ the common representative

Name and Address	State of Nationality	State of Residence
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☒ the person ☒ the name ☒ the address ☒ the nationality ☒ the residence

Name and Address	State of Nationality	State of Residence
THE UNIVERSITY COURT OF THE UNIVERSITY OF GLASGOW No. 2 The Square University Avenue Glasgow G12 8QQ United Kingdom	GB	GB
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

3. Further observations, if necessary:
The above-named applicant has been added to the record as co-applicant for all designated States except the United States of America.

4. A copy of this notification has been sent to:

☒ the receiving Office
☐ the International Searching Authority
☐ the International Preliminary Examining Authority
☐ the designated Offices concerned
☒ the elected Offices concerned
☐ other:

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Ann Bardini
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 730.91.11

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: GARY COLBY
PANITCH SCHWARZE JACOBS & NADEL
ONE COMMERCE SQUARE
2005 MARKET STREET, 22ND FLOOR
PHILADELPHIA, PA 19103-7086

FILE COPY 408

WRITTEN OPINION

(PCT Rule 66)

Form PCT/IPEA/408 (cover sheet) (July 1998) DO NOT MAIL

	Date of Mailing *(day/month/year)*
Applicant's or agent's file reference 9596-83PC	**REPLY DUE** within **TWO** months from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US99/05466	12 MARCH 1999	12 MARCH 1998

International Patent Classification (IPC) or both national classification and IPC
IPC(7): C12N 1/20, 5/00, 15/00; D21C 1/00 and US Cl.: 435/252.3, 277.1, 320.1, 325

Applicant
The Trustees of the University of Pennsylvania

1. This written opinion is the first (first, etc.) drawn by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

- I [X] Basis of the opinion
- II [ ] Priority
- III [ ] Non-establishment of opinion with regard to novelty, inventive step or industrial applicability
- IV [X] Lack of unity of invention
- V [X] Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI [ ] Certain documents cited
- VII [ ] Certain defects in the international application
- VIII [ ] Certain observations on the international application

3. The applicant is hereby invited to reply to this opinion.

When? See the time limit indicated above. ~~The applicant may, before the expiration of that time limit, request this Authority to grant an extension., see Rule 66.2(d).~~

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 *bis*.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 12 JULY 2000.

Facsimile No.	Authorized officer AND Telephone No.
(703) 305-3230	SUSAN UNGAR (703) 308-0196

WRITTEN OPINION
Form PCT/IPEA/408 (Box I) (July 1998)
FILE COPY - DO NOT MAIL

International application No.
PCT/US99/05466

I. Basis of the opinion

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-46, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

[X] the claims:
pages 47-49, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

[X] the drawing:
pages NONE, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

[X] the sequence listing part of the description:
pages NONE, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the written opinion was drawn on the basis of the sequence listing:

[ ] contained in the international application in printed form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [ ] This opinion has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*

WRITTEN OPINION
Form PCT/IPEA/408 (Box II) (July 1998)
FILE COPY - DO NOT MAIL

International application No.
PCT/US99/05466

II. Priority

1. ☐ This opinion has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been fo und invalid.

Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

WRITTEN OPINION

Form PCT/IPEA/408 (Box III) (July 1998)
FILE COPY - DO NOT MAIL

International application No.
PCT/US99/05466

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application.

☐ claims Nos. _

because:

☐ the said international application, or the said claim Nos. _ relate to the following subject matter which does not require international preliminary examination *(specify)*.

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. _ are so unclear that no meaningful opinion could be formed *(specify)*.

☐ the claims, or said claims Nos. _ are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for said claims Nos. _.

2. A written opinion cannot be drawn due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

WRITTEN OPINION

Form PCT/IPEA/408 (Box IV) (July 1998)
FILE COPY - DO NOT MAIL

International application No.
PCT/US99/05466

IV. Lack of unity of invention

1. In response to the invitation (Form PCT/IPEA/405) to restrict or pay additional fees the applicant has:

- [ ] restricted the claims. (See Supplemental Sheet)
- [X] paid additional fees.
- [ ] paid additional fees under protest.
- [ ] neither restricted nor paid additional fees.

2. This Authority found that the requirement of unity of invention is not complied with for the following reasons and chose, according to Rule 68.1 not to invite the applicant to restrict or pay additional fees:

3. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this opinion:

- [X] all parts.
- [ ] the parts relating to claims Nos. .

WRITTEN OPINION

Form PCT/IPEA/408 (Box V) (July 1998)
FILE COPY - DO NOT MAIL

International application No.

PCT/US99/05466

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	1-24 and 26-29	YES
	Claims	25	NO
Inventive Step (IS)	Claims	16 and 23	YES
	Claims	1-15 and 17-22 and 24-29	NO
Industrial Applicability (IA)	Claims	1-29	YES
	Claims	NONE	NO

2. citations and explanations

Claims 1-15, 17-22, 24-29 lack an inventive step under PCT Article 33(3) as being obvious over US Patent No. 5,601,818 in view of US Patent No. 5,585,096.

The claims are drawn to a producer cell for administration to a subject having tumor cells said producer cell comprising an oncolytic virus which is capable of replicating in said producer cell, said producer cell being incapable of sustained survival in the body of the subject (Claim 1), wherein the oncolytic virus is cytotoxic with respect to said producer cell in the body of the subject (Claim 2), wherein said producer cell is rendered incapable of sustained survival in the body of the subject by exposing said producer cell to a lethal dose of radiation (claim 3), wherein said lethal dose of radiation is a dose which enhances the burst size of said producer cell (claim 4), wherein the dose is about 3 Gray (claim 5), wherein said producer cell has a suicide gene (claim 5), selected from the group including thymidine kinase (claim 7), wherein the producer cell exhibits binding affinity for a tumor cell in the subject (claim 8), wherein the tumor cell is an epithelial tumor cell (claim 9), an epithelial ovarian cancer cell (claim 10), wherein said oncolytic virus is capable of replicating in a tumor cell of the subject (claim 11), wherein said oncolytic virus is less capable of replicating in a non-tumor cell of the subject than in a tumor cell (claim 12), wherein the oncolytic virus is incapable of replicating in a non-tumor cell of the subject (claim 13), wherein the replication of said oncolytic virus is under the control of a tumor-associated transcriptional promotor (claim 14), wherein the promoter is PSA promoter or TGF beta promoter (claim 15), wherein said producer cell is selected from a group including a PA-1 (claim 17), wherein said producer cell is PA-1 cell, (claim 18), Wherein the virus is selected from the group including HSV-1, HSV-2, adenovirus(claim 19), wherein the HSV-1 does not express functional ICP34.5 (claim 20), wherein the HSV-1 is selected from a group of HSV viruses including G207(claim 21) wherein the HSV-2 is selected from a group (claim 22), wherein the producer cell further comprises a nucleic acid encoding a cytokine (claim 24), an anti-tumor agent comprising a producer (Continued on Supplemental Sheet.)

International application No.
PCT/US99/05466

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

Application No. Patent No.	Publication Date *(day/month/year)*	Filing Date *(day/month/year)*	Priority date (valid claim) *(day/month/year)*

2. Non-written disclosures (Rule 70.9)

Kind of non-written disclosure	Date of non-written disclosure *(day/month/year)*	Date of written disclosure referring to non-written disclosure *(day/month/year)*

International application No.
PCT/US99/05466

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

International application No.
PCT/US99/05466

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

TIME LIMIT:

The time limit set for response to a Written Opinion may not be extended. 37 CFR 1.484(d). Any response received after the expiration of the time limit set in the Written Opinion will not be considered in preparing the International Preliminary Examination Report.

IV. LACK OF UNITY OF INVENTION:

1. This response is made to a telephone Lack of Unity requirement (see telephone memorandum attached hereto or attached to a prior Written Opinion).

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):

cell which comprises TK which exhibits binding affinity for a tumor cell in a human patient, is incapable of sustained survival in the body of the patient, is administered to the patient, binds with a tumor cell in the patient, wherein gancyclovir is thereafter administered, wherein said mammalian cell metabolizes gancyclovir to generate a cytotoxic metabolite which is provided to the tumor cell with which the mammalian cell has bound (claim 25), a method of killing tumor cells comprising administering a producer cell comprising an oncolytic virus wherein said oncolytic virus is capable of replicating in said producer cell, said producer cell being incapable of sustained survival in the body of the mammal (claim 26), wherein the mammal is a human afflicted with an epithelial cancer (claim 27), wherein said mammal is a human afflicted with a tumor (claim 28), use of a producer cell for manufacture of a medicament for administration to a patient having tumor cells (claim 29).

US Patent No. 5,601,818 teaches a producer cell for administration to a subject having tumor cells comprising a myelomonocyte tumor cell (col 10, line 62) , a fibrosarcoma cell (col 11, line 51), a human colon carcinoma cell (col 12, line 21), a mammary tumor cell (col 13, line 50), a neuroblastoma cell (col 16, line 14) and specifically teaches that any cell type can act as a recipient for insertion of the selected gene or genes (col 5, lines 63-65) and teach that the cells are transformed to comprise at least one foreign gene (col 4, lines 29-40), a suicide gene herpes simplex virus type I thymidine kinase wherein the expression of this gene renders the producer cell susceptible to eradication by gangiclovir, an anti-viral nucleoside analogue (para bridging cols 4 and 5) which is metabolized by the cell to produce a cytotoxic byproduct, wherein the producer cell further comprises a cytokine gene (col 5, lines 31-52), wherein the foreign gene, comprising HSVI-TK is under the control of a tumor-associated transcriptional promoter, SV40 (col 4, lines 40-45), wherein the live cancer producer cell is rendered incapable of sustained survival by exposing the cell to a lethal dose of radiation (col 7, lines 53-57), and teaches that the present invention can be widely utilized for the treatment of human cancers including melanoma, colon cancer ovarian cancer, stomach cancer, neuroblastoma, squamous cell carcinoma (col 8, lines 3-15) and specifically teaches that an aspect of the invention is the homing of producer cells to parental cancer cells in vivo and teaches that the ability of malignant cells to migrate or metastasize is the phenomenon whereby cells of the same type tend to aggregate or grow together both in vitro and in vivo and teach that the producer cells appear to migrate to parental cells resident in the subject and after initiation of therapy lead to rapid killing of the parental cells by the subject (col 10, lines 11-39).

US Patent No. 5,601,818 teaches as set forth above but does not teach a producer cell comprising an oncolytic virus which is capable of replicating in said producer cell wherein said oncolytic virus is cytotoxic with respect to said producer cell, a dose of radiation that enhances the burst size of said producer cell, wherein the dose is about 3 Gray, does not teach a producer cell wherein the producer cell has an affinity for an epithelial ovarian cancer cell wherein said oncolytic virus is capable of replicating in a tumor cell of the subject, wherein said oncolytic virus is less capable of replicating in a non-tumor cell of the subject than in the tumor cell, wherein the oncolytic virus is incapable of replicating in a non-tumor cell, wherein the tumor associated transcriptional promoter is PSA promoter or TGF beta promoter, wherein said producer cell is selected from the group including PA-1, wherein the virus is HSV-1, HSV-2 or adenovirus, wherein the HSV-1 does not express functional ICP34.5, does not teach a method of killing tumor cells comprising administering a producer cell comprising an oncolytic virus wherein said oncolytic virus is capable of replicating in said producer cell wherein said producer cell is incapable of sustained survival in the body of a mammal or the use of said producer cell for manufacture of a medicament for administration to a patient having tumor cells.

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

US Patent No. 5,585,096 teaches a replication-competent viral vector suitable for use in humans that is capable of killing human tumor cells in vivo, that exhibits hypersensitivity to anti-viral agents and an inability to revert to wt virus (col 3, lines 105), wherein the virus is a replication-competent herpes simplex virus that is incapable of expressing a functional ICP34.5 (col 3, lines 33-40) wherein the herpes simplex virus is an HSV1 G207 (col 4, lines 60-65) and teaches that kinds of cells that can be killed pursuant to the present invention include melanoma cells, pancreatic cancer cells, prostate carcinoma cells, breast cancer cells, lung cancer cells (col 3, lines 52-58). The present invention exploits the ability of mutant replication-competent HSV-1 to enter a tumor cell in situ, make multiple copies, lyse the tumor cell and spread to additional tumor cells with relatively minor effects on surrounding normal cells (col 5, lines 14-27) and teaches that DNA constructs employing HSV-2 are encompassed by the present invention (col 8, lines 6-10). It is noted that HSV-1 comprises thymidine kinase.

Lust et al (Current Topics in Microbiology and Immunology, 1995, 194:199-206) teaches that PA-1, a well known epithelial ovarian cancer cell, can be transfected with foreign DNA and express said DNA (see abstract).

It would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made to substitute the PA-1 cells of Lust et al for the producer cells of US Patent No. 5,601,818 because US Patent No. 5,601,818 specifically teaches that any cell can be substituted for the producer cells of the invention and because US Patent No. 5,601,818 specifically teaches that the producer cells of the invention migrate to parental cells resident in the subject and after initiation of therapy lead to rapid killing of the parental cells by the subject. One would have been motivated to substitute the PA-1 cells of Lust et al for the producer cells of US Patent No. 5,601,818 because the cells are epithelial ovarian cancer cells and because US Patent No. 5,601,818 specifically teaches that the invention is useful for treating ovarian cancer. Further, it would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made to substitute the HSV-1 or HSV-2 construct of US Patent 5,585,096 for the foreign gene construct of US Patent No. 5,601-818 because US Patent No. 5,858,096 specifically teaches that the invention exploits the ability of mutant replication-competent HSV-1 to enter a tumor cell in situ, make multiple copies, lyse the tumor cell and spread to additional tumor cells with relatively minor effects on surrounding normal cells. It is clear that the migration of the PA-1 producer cells to the site of the tumor will facilitate treatment of the tumors. Further, it would be expected that a lethal dose of radiation would have to be at least 3 Grays and that the lethal dose would be expected to enhance the burst size of the producer cell. Further, it is known that PSA promoter and TGF beta promoter are conventionally used in virus vectors.

Claims 16 and 23 the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest a producer cell wherein the oncolytic virus is incapable of replicating in any cell of the subject, a producer cell wherein the oncolytic virus is an adenovirus.

Claim 25 lacks novelty under PCT Article 33(2) as being anticipated by US Patent No. 5,602,818.

The claim is drawn to TK which exhibits binding affinity for a tumor cell in a human patient, is incapable of sustained survival in the body of the patient, is administered to the patient, binds with a tumor cell in the patient, wherein gancyclovir is thereafter administered, wherein said mammalian cell metabolizes gancyclovir to generate a cytotoxic metabolite which is provided to the tumor cell with which the mammalian cell has bound.

US Patent No. 5,602,818 teaches as set forth above, all of the limitations of the claim are met.

---------- NEW CITATIONS ----------

LUST et al. Sequence, expression and function of an nRNA encoding a soluble form of the human interleukin-6 receptor. Current Topics in Microbiology and Immunology. 1995. Vol 194 pages 199-206, see abstract.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

REC'D 17 OCT 1996
WIPO PCT

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
MGH/YF/P06806PC		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB 95/ 01791	28/07/1995	29/07/1994
International Patent Classification (IPC) or national classification and IPC		
A61K35/76		
Applicant		
MEDICAL RESEARCH COUNCIL et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consists of a total of 4 sheets.

3. This report contains indications and corresponding pages relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
27/02/1996	14.10.96
Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	L. Olsen Telephone No.

Intern. application No.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT PCT/GB95/01791

I. Basis of the report

1. This report has been drawn up on the basis of (Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.):

[ ] the international application as originally filed.

[x] the description, pages 1-57, as originally filed,
pages _____, filed with the demand,
pages _____, filed with the letter of _____,
pages _____, filed with the letter of _____,

[x] the claims, Nos. _____, as originally filed,
Nos. _____, as amended under Article 19,
Nos. _____, filed with the demand,
Nos. 1-19, filed with the letter of 16.08.96,
Nos. _____, filed with the letter of _____,

[x] the drawings, sheets/fig 1/9 - 9/9, as originally filed,
sheets/fig _____, filed with the demand,
sheets/fig _____, filed with the letter of _____,
sheets/fig _____, filed with the letter of _____.

2. The amendments have resulted in the cancellation of:
[ ] the description, pages _____.
[ ] the claims, Nos. _____.
[ ] the drawings, sheets/fig _____.

3. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims 1-19	YES
	Claims	NO
Inventive Step (IS)	Claims 11,19	YES
	Claims 1-10,12-18	NO
Industrial Applicability (IA)	Claims 12-15	YES
	Claims 1-11,16-19 ?	NO

2. CITATIONS AND EXPLANATIONS

1. The following documents have been considered for the purposes of this report:

D1= Neurosurgery, **32** (1993) p.597-603;
D2= Science, **250** (1990) p.1262-1266;
D3= WO 92/13943;
D4= Gene Therapy, **1** (Sep. 1994) p.323-331.

2. Although document D4 does not constitute prior art within the meaning of Rule 64.1(b) PCT (the present claimed priority is assumed to be valid), it appears to disclose features of claims 1-19.

3. The present application does not satisfy the criterion set forth in Article 33(3) PCT because the subject-matter of claims 1-10 and 12-18 does not involve an inventive step (Rule 65(1)(2) PCT).

The document D1 discloses the use of a herpes simplex

virus-1 (HSV-1) strain F mutant (R3616) as an anti-brain tumour agent in an in vivo mouse model. The purpose of this model is to simulate a treatment of human brain tumours. R3616 has a 1 kb deletion in a Bam H1 restriction fragment of the R_L terminal repeat. R3616 is shown to be non-neurovirulent. R3616 is furthermore disclosed in D2.

As D1 teaches the use of mutated and non-neurovirulent HSV-1 as an anti-cancer agent, it would be obvious to try the effect of other mutated and non-neurovirulent HSV-1 viruses. D3 discloses such a HSV-1 strain, namely the mutant HSV-1 strain 17 (1716), which is modified in the R_L terminal repeat within a Bam H1 restriction fragment (a deletion of 759 bp). Strain 1716 is used as a vaccine according to D3, for which purpose the strain has to be non-neurovirulent. The HSV-1 strain 17 containing a mutation in the R_L terminal repeat, e.g. strain 1716, would thus be an obvious alternative to the mutant strains disclosed in D1 for use as an anti-cancer agent.

The use of such advantageous mutated viruses in treating cancer in a mammal, e.g. in a human, is thus suggested in D1 and D3 taken together with D1. The subject-matter of claims 1-10 and 16-18 is therefore obvious in the light of D1 taken together with D3. Furthermore, the use of the mutated viruses in the manufacture of a medicament for treating tumours (claims 12-15) also appears to be obvious.

4. The subject-matter of claims 1-10 and 16-18 appears to be novel over the cited prior art as the HSV-1 strain 17 is not previous disclosed as being useful in cancer treatment. The subject-matter of claims 11 and 19 appears to be novel and to involve an inventive step as a succesful treatment of secondary metastatic cancer of the central nervous system is neither disclosed in nor

obvious from the cited prior art. The subject-matter of claims 12-15 appears to be novel as the medicament is either comprising the mutated HSV-1 strain 17 (when refering to claims 1-10) or for use in treating a secondary metastatic tumour of the central nervous system (when refering to claim 11).

5. For the assessment of the present claims 1-11 and 16-19 on the question whether they are industrially applicable, no unified criteria exist in the PCT. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but will allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

CLAIMS

1. Use as an anti-cancer agent of a mutant herpes simplex virus type HSV-1 wherein the mutant virus is a mutant strain 17 virus and comprises a modification in the γ34.5 gene in the long repeat region (R_L) such that the γ34.5 gene is non-functional.

2. Use of a mutant herpes simplex virus according to claim 1 wherein the virus is substantially non-neurovirulent.

3. Use of a mutant herpes simplex virus according to any preceding claim wherein the modification to the virus is made within the Bam H1 s restriction fragment of the R_L terminal repeat.

4. Use of a mutant herpes simplex virus according to claim 3 wherein the modification is a deletion of from 0.1 to 3kb, in particular of from 0.7 to 2.5 kb.

5. Use of a mutant herpes simplex virus according to claim 4 wherein the deletion is a 759 bp deletion in the γ34.5 gene.

6. Use of a mutant herpes simplex virus according to any of the preceding claims as an anti-brain tumour agent.

AMENDED SHEET

7. Use of a mutant herpes simplex virus according to claim 6 against primary tumours originating within the brain and nervous system.

8. Use of a mutant herpes simplex virus according to claim 6 against metastatic tumours, in particular against metastases of melanoma cancers.

9. Use of a mutant herpes simplex virus according to any of the preceding claims wherein the use is in a mammal, in particular in a human.

10. Use according to any of the preceding claims wherein the mutant herpes simplex virus is mutant 1716.

11. Use as an anti-cancer agent of a mutant herpes simplex virus type HSV-1 wherein the mutant virus comprises a modification in the γ34.5 gene in the long repeat region (R_L) such that the γ34.5 gene is non-functional; the anti-cancer use being in respect of cancer of the central nervous system including the brain, the cancer being a secondary metastatic tumour.

12. Use of a mutant herpes simplex virus according to any preceding claim in the manufacture of a medicament for the treatment of cancer in mammals, in particular in humans.

13. Use of a mutant herpes simplex virus according to claim 12 in the manufacture of a medicament for the treatment of brain tumours in mammals, in particular in humans.

14. Use of a mutant herpes simplex virus according to claim 12 or claim 13 in the manufacture of a medicament for the treatment of primary tumours originating within the brain and/or nervous system.

15. Use of a mutant herpes simplex virus according to any of claims 12 to 14 in the manufacture of a medicament for the treatment of metastatic tumours, in particular against metastases of melanoma cancers.

16. A method of treating cancer in mammals, in particular in humans by administering a pharmaceutical formulation comprising a mutant herpes simplex virus type HSV-1 wherein the mutant virus is a mutant strain 17 virus and comprises a modification in the γ34.5 gene in the long repeat region (R_L) such that the γ34.5 gene is non-functional.

17. A method of treating cancer in mammals, in particular in humans according to claim 16 by administering a pharmaceutical formulation directly into the tumour.

18. A method of treating cancer in mammals, in particular in humans according to claim 16 by administering a pharmaceutical formulation parenterally into the blood stream feeding the tumour.

19. A method of treating cancer in mammals, in particular in humans, by administering a pharmaceutical formulation comprising a mutant herpes simplex virus type HSV-1 wherein the mutant virus comprises a modification in the γ34.5 gene in the long repeat region (R_L) such that the γ34.5 gene is non-functional; the cancer being a cancer of the central nervous system including the brain which is a secondary metastatic cancer tumour.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB95/01791	28/07/95	29/07/94
Applicant		
MEDICAL RESEARCH COUNCIL et al.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [ ] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [ ] filed with the international application.
   - [ ] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] Transcribed by this Authority

4. With regard to the **title,**
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. 3
   - [ ] as suggested by the applicant.
   - [X] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.
   - [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/[redacted]B 95/01791

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K35/76 C12N15/38

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NEUROSURGERY, vol. 32, 1993 pages 597-603, MARKERT, J.M. ET AL. cited in the application see the whole document ---	1-16
A	WO-A-92 13943 (SMITH-KLINE BEECHAM BIOLOGICALS S.A.) 20 August 1992 cited in the application see the whole document ---	1-16
A	SCIENCE, vol. 250, 1990 pages 1262-1266, CHOU, J. ET AL. cited in the application see abstract ---	1-16
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
1 February 1996	14. 03. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Olsen, L

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF GENERAL VIROLOGY, vol. 70, 1989 pages 705-716, TAHA, M.Y. ET AL. see the whole document ---	1-16
P,X	GENE THERAPY, vol. 1, September 1994 pages 323-331, BOVIATSIS, E.J. ET AL. see the whole document -----	1-16

INTERNATIONAL SEARCH REPORT

Information on patent family members

Application No PCT/GB 95/01791

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9213943	20-08-92	AU-B-	657731	23-03-95
		AU-B-	1182992	07-09-92
		EP-A-	0571410	01-12-93
		JP-T-	6507066	11-08-94
		NZ-A-	241476	23-12-93
		NZ-A-	245842	23-12-93

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 35/76, C12N 15/38

A2

(11) International Publication Number: WO 96/03997

(43) International Publication Date: 15 February 1996 (15.02.96)

(21) International Application Number: PCT/GB95/01791

(22) International Filing Date: 28 July 1995 (28.07.95)

(30) Priority Data:
9415320.2 29 July 1994 (29.07.94) GB

(71) Applicants *(for all designated States except US)*: MEDICAL RESEARCH COUNCIL [GB/GB]; 20 Park Crescent, London W1N 4AL (GB). THE WISTAR INSTITUTE [US/US]; 3601 Spruce Street, Philadelphia, PA 19104-4268 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: MACLEAN, Alasdair, Roderick [GB/GB]; 36 Colwood Place, Glasgow G53 7YB (GB). BROWN, Susanne, Moira [GB/GB]; 5 Bellshaugh Road, Glasgow G12 0SN (GB). FRASER, Nigel, William [GB/US]; 511 South 46th Street, Philadelphia, PA 19143 (US). RANDAZZO, Bruce, Paul [US/US]; 2409 Manning Street, Philadelphia, PA 19103 (US).

(74) Agents: McCALLUM, William, Potter et al.; Cruikshank & Fairweather, 19 Royal Exchange Square, Glasgow G1 3AE (GB).

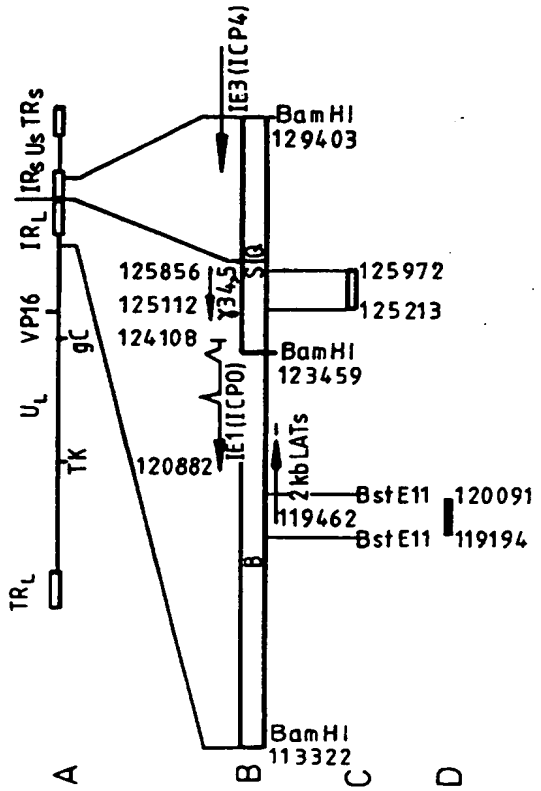
(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: TREATMENT OF CANCER USING HSV MUTANT

(57) Abstract

Use as an anti-cancer agent of a mutant herpes simplex virus wherein the mutant virus comprises a modification in the γ34.5 gene in the long repeat region (R_L) such that the γ34.5 gene is non-functional, manufacture of medicaments and methods of testing cancer in mammals employing HSV mutant.



TREATMENT OF CANCER USING HSV MUTANT

FIELD OF INVENTION

The present invention relates to the use of a herpes simplex virus (HSV) mutant for the treatment of cancer tumors, particularly those of the brain or nervous system whether the tumors are metastatic tumors or primary tumors.

BACKGROUND

The DNA sequence of herpes simplex type 1 (HSV-1) is known (references 13,25) and is linear with a length of about 152k residues. It consists of two covalently linked segments, designated long (L) and short (S). Each segment contains a unique sequence flanked by a pair of inverted terminal repeat sequences. The long repeat (R_L) and short repeat (R_S) are distinct. The unique long (U_L) region includes genes UL1 to UL56, and the U_S region includes genes US1 to US12.

A relatively large number of patients with advanced cancers will develop metastatic lesions in the brain and spinal cord. This frequently results in severe and debilitating neurological complications including headache, paralysis, seizures, and impaired cognition. It has been estimated that 70,000 cancer deaths occur each year in the United States with metastatic lesions to the central nervous system

(CNS). Radiation and steroids are presently the principle therapies used, however, they are only palliative, and frequently cause significant neuropsychological and endocrinological morbidity. Surgery is generally reserved for removal of solitary metastases, and is often not curative (1).

Viral therapy for the destruction of tumors is not a new concept. Effects in various experimental tumor systems have been demonstrated using parvovirus H-1, Newcastle disease virus, retroviral vectors containing drug susceptibility genes, and Herpes Simplex Type I virus (HSV-1) (2-7). The mechanisms by which viruses improve the outcome in experimental tumor systems are complex and poorly understood. Brain tumors represent a dividing cell population occurring within an essentially non-dividing cell population of support cells, and terminally differentiated neurons. Thus, in the context of brain tumor therapy, one rationale is to select a virus that replicates exclusively or preferentially in dividing cells. Such a virus may be capable of establishing a lytic infection exclusively in tumor cells within the CNS, ultimately destroying the tumors without infecting surrounding brain, and without deleterious effects to the host.

Pioneering experiments with HSV showed a dose dependent improvement in survival of nude mice bearing intracranial human gliomas following intratumoral

therapy with mutant HSV-1 dlspTK (3). This virus has a deletion in the viral thymidine kinase (TK) gene, (8) and exhibits a relatively neuro-attenuated phenotype in mice (9). However, dlspTK infection of tumor bearing animals causes histologically evident encephalitis (3). The use of TK⁻ mutants of HSV-1 for viral therapy also has an inherent major disadvantage in that these viruses are resistant to the clinically effective anti-viral agents acyclovir and ganciclovir (10).

The terminal 1 kb of the long repeat region (R_L) of the HSV-1 and HSV-2 genomes contain a gene (11-13), that confers neurovirulence. Deletion or mutation of this gene (γ34.5), results in variants that grow as well as wild type virus on dividing cells of many established cell lines, but show impaired replication on non-dividing cells (12-14). In mice, γ34.5 null mutants are incapable of replicating in the central nervous system, and do not cause encephalitis (12,15-16).

A mutant HSV-1 called R3616, containing a 1000 base pair (bp) deletion in γ34.5, with an LD_{50} (minimum dose of virus that kills 50% of infected animals) that is at least $3x10^3$ fold greater than wild type F strain virus from which it was derived (12), has been shown to improve the outcome of nude mice bearing intracranial human gliomas (17). In the work presented here, we have utilized an HSV-1 strain 17

mutant virus called 1716, that has a 759 bp deletion in γ34.5 (16).

The construction of mutant virus 1716 is described in published patent application W092/13943 (PCT/GB92/00179) the contents of which are incorporated herein by reference. However, this patent publication is solely concerned with the use of mutant 1716 as a vaccine, either in itself or as a vector vaccine which includes a heterologous gene coding for an antigen.

Melanoma is a prevalent malignancy. Cerebral metastases occur in up to 75% of patients with metastatic disease, and are among the most common causes of death (18-22). Presently, the life span of patients with CNS melanoma is short, ranging from 2 to 7 months (23).

It is an object of the present invention to provide an improved HSV-based viral therapy of cancer tumors.

STATEMENT OF INVENTION

The present invention in one aspect provides the use as an anticancer agent of a mutant herpes simplex virus which has been modified in the γ34.5 gene of the long repeat region (R_L) such that the gene is non-functional.

The invention also relates to a method of treatment of cancer in a mammal (human or animal) by

the administration to the mammal of an anti-cancer effective dose of the mutant herpes simplex virus.

DETAILED DESCRIPTION

For the purposes of the present invention "non-functional" means that the gene has been modified by deletion, insertion or substitution (or other change in the DNA sequence such as by rearrangement) such that it does not express the normal product or a functionally equivalent product. The effect of the non-functionality of the gene is that the neurovirulence of the virus to the patient is substantially removed.

Thus the invention is based on the finding that rendering the γ34.5 gene non-functional provides an HSV mutant which is particularly effective in destroying dividing tumor cells, whilst at the same time the HSV mutant does not replicate within normal non-cancerous cells. It therefore has the potential to provide a safe anti-cancer treatment.

Two types of herpes simplex virus are known HSV-1 and HSV-2 and either may be employed in the present invention to provide the HSV mutant. Inter-type recombinants containing DNA from both types could also be used.

The modification may be effected at any convenient point within the γ34.5 gene, and such point generally corresponds to a restriction enzyme site.

The modification may be within the Bam H1 s restriction fragment of the R_L terminal repeat (corresponding to 0-0.02 and 0.81 - 0.83 mu). The modification is typically a deletion of 0.1 to 3kb, particularly 0.7 to 2.5kb. In this work a 759 bp deletion in γ34.5 was made in the HSV-1 mutant, referred to as 1716.

The HSV genome also includes a number of other genes which are non-essential to the successful culturing of the virus. It is, of course, necessary to retain within the HSV mutant the ability to culture the mutant so that the mutant is self-replicating and stocks of the mutant can be grown in tissue culture. Lethal modifications of the genome which remove the ability to culture the HSV mutant are not acceptable.

However, in addition to the primary modification to the γ34.5 gene of the R_L region, it may be advantageous to also include in the HSV mutant one or more secondary non-lethal modifications within non-essential genes.

The present invention also encompasses as a new product an HSV mutant which includes in addition to the primary modification, a secondary non-lethal modification (for example within Vmw65). The mutant may be derived from HSV-1 or HSV-2.

In a similar way, other secondary modifications may involve modification of the latency associated transcript (LAT) promoter so as to render the promoter

non-functional and prevent transcription thereof.

Herpes simplex virus infects the brain and nervous system. The HSV mutant is effective against primary tumors originating within the brain and nervous system, but is particularly useful against metastatic tumors where cancer cells originating elsewhere have lodged in the brain or nervous system (particularly the central nervous system (CNS)). Brain metastases occur commonly in a variety of human cancers (e.g. melanomas),and at present such cases are invariably fatal. The efficacy of treatment according to the invention employing the HSV mutant will depend on the time after origination of the tumor at which the treatment is initiated, but efficacy is improved by early treatment for example in 1 to 30 days.

The LD_{50} (minimum dose of virus that kills 50% of infected animals) of the 1716 mutant in respect of mice is 10^6 fold greater than that of the wild type 17+ virus from which it is derived. Thus the neurovirulence of 1716 is essentially removed relative to the wild type virus.

The effective non-toxic dose of HSV mutant can be determined by routine investigation by the skilled addressee, and will depend on a number of factors including the particular species of mammal and the extent of development of the tumor. A guide can be obtained from the Examples herein.

In a further aspect of the invention there is

provided a method of treating cancer in mammals, in particular in humans by administering a pharmaceutical formulation comprising the HSV mutant to mammals, in particular to humans. Thus, the method of treatment can comprise the administration of a pharmaceutical formulation comprising the HSV mutant by injection directly into the tumour or parenterally into the blood stream feeding the tumour.

It will usually be presented as a pharmaceutical formulation including a carrier or excipient, for example an injectable carrier such as saline or apyrogenic water. The formulation may be prepared by conventional means.

Embodiments of the invention will now be described by way of example only.

Figures 1 and 2 show the results of experiments described fully in Examples 3 and 5 respectively.

Figure 1: Survival Curves

Tumor-bearing mice injected at 10 days (Figure 1b) and at 5 days (Figure 1b) post tumor injection with HSV-1 mutant 1716.

Figure 2: Relative replication rates of HSV-1 mutant

Relative replication rates of HSV-1 mutant 1716 in brain tumor (closed squares); and of 1716 and wild type 17+ in non-tumor brain (open squares and closed triangles respectively).

Figur 3: HSV-1 Genome map.

HSV-1 genome showing approximate location of the

γ34.5, 2kb latency associated transcript (LAT) and neighbouring genes. A. The 152kb HSV-1 strain 17+ genome, illustrating the unique long and short segments of the genome, U_L and U_S (lines), bounded by internal (IR) and terminal repeat (TR) regions (open boxes). Hatch marks show location of the VP16, thymidine kinase (TK) and glycoprotein C (gC) genes. B. Expanded view of the U_L/U_S region of the genome the location of the γ34.5, ICPO and ICP4 mRNAs and the location of the 2.0kb LAT which is expressed during acute and latent infection. C. The location of the 759 bp deletion in strain 1716. D. The location of the LAT specific BstEII-BstEII probe used for in situ detection of HSV specific gene expression. Nucleotide positions are based on DNA sequence analysis of Perry and McGeoch (45).

Figure 4: Quantification of infectious virus in nude mouse brain after IC inoculation.

To investigate the extent of strain 1716 replication in brain tumors, nude mice were injected with NT2 cells. Twelve days later each mouse was infected with 5×10^5 PFU of strain 1716 at the same stereotactic coordinates (open circles). At the times indicated, mice were sacrificed, the brains were frozen in Liquid N_2 and stored at -70°C. Specimens were thawed rapidly, homogenized, and viral titration was performed in triplicate on BHK cells. To establish the growth characteristic of strain 1716 and parental 17^+ in

brain without tumor, mice were injected intracranially with either 5 x 10^4 PFU of 1716 (closed circles) or 1 x 10^6 PFU of 17^+ (closed triangles). Mice were sacrificed at the times shown and processed as described in Methods. Each point is the mean of 2 mice with SEM bars.

Figure 5: Detection of replicating virus by immunohistochemistry and in situ hybridization

Nude mice were IC injected with 3 x 10^4 NT2 cells. 14 days later they were inoculated with 5 x 10^5 PFU of 1716. A. Control mice with tumor after 14 days. B. Tumor is histologically very diverse, arrows: tubular structures. C. MOC-1 antibody specifically identifies the NT2 tumor cells. D. Antibody MIB-1 identifies cycling cells. Note low number of labeled cells in tubular structures (asterisk). E. 14 day tumor after three days infection with strain 1716. The arrow indicates a region of extensive tumor lysis and necrosis. F. Infected cells show characteristic features of herpes infected cells such as intranuclear inclusion bodies formation, cytomegaly and necrosis. G. Herpes antigen is limited to tumor cells. H. Virus replicates in tumor cells at interface between tumor and host brain shown using anti-HSV antibodies. I. The kinetics of viral replication are delayed at 3 days after infection in the tubular structures compared to surrounding non-tubular cells as shown with anti-HSV antibodies. The arrow identifies a rare

HSV antigen positive tubular cell. These tubular structures are lysed at later days after infection. J. Herpes gene expression is also limited to tumor by in situ hybridization (black grains). K and L. Wild-type virus (17^+) replicates in brain and tumor and spreads throughout the whole brain. M. H&E of 14 day tumor 18 days after infection with strain 1716. Note the small size of tumor. N. Viral antigen and O. viral gene expression is strikingly limited to the residual tumor mass. Abbreviations: T=tumor; B=host brain

(Scale Bar:=1.2mm in A; =62.5μm in B,C,D; =2.0mm in E&G ; =12μm in F; = 450μm in H, M, N, O; =90μm in I; =200μm in J: =900μm in K and L).

Figure 6: MRI analysis of treated and untreated NT2 tumors.

Nude mice were injected stereotactically with 3 x 10^4 NT2 cells and 11 days later (d11 post-tumor cell implantation) T1 weighted, gadolinium-enhanced MRIs (A,E) were performed. The presence of a tumor (T) is confirmed by the white enhancing lesion appearing in the superior right hemisphere in these mice. These sections show the area of the maximal tumor mass in cross section. The following day these mice were inoculated with either 5x10^5 PFU of strain 1716 or culture medium. In control mice, the tumor progressed over time and IC volume increased dramatically (B: day 32 post-tumor: C & D: day 41 post-tumor). In strain

1716 treated mice the tumor regressed and showed no evidence of live tumor cells or virus in the brain (F & G: day 36 post-tumor), (Scale Bar: = 2.77mm in D & G).

Figure 7: Electronmicroscopy

Tumors from nuce mice which were mock inoculated or inoculated with strain 1716 were harvested and processed for electronmicroscopy. A: Electronmicrograph of uninfected tumor cells. B: EM of NT2 tumor cells early in infection DNA condensation and viral domains (open arrow) can be observed; C: NT2 tumor cells late in infection, marginated chromatin (*) and viral particles (arrowhead) can be observed, D: A dividing cell that is infected, arrow: nuclear membrane, c: cytoplasm, n: nucleus (original magnifications at 2500x).

Figure 8: Prolonged survival of NT2 tumor bearing mice treated with strain 1716.

A: Survival Experiment (Table 1, Study V)- 20 nude mice were stereotactically injected with $3x10^4$ NT2 cells. Twelve days later, 10 mice were sterotactically injected with $5x10^5$ PFU/5μl of strain 1716 (treated, closed circle) and 10 mice were mock injected with 5μl of viral culture medium (mock, closed triangle). B: Survival Experiment (Table 1, Study VI)- 17 nude mice were stereotactically injected with $3x10^4$ NT2 cells. Ten days later, 9 mice were stereotactically injected $5x10^5$ PFU/5μl of strain 1716

(treated, closed circle) and 8 mice were mock injected with 5μl of viral culture medium (mock, closed triangle). C: Weight Graph- Weights of control (closed triangle) and treated mice (closed circles) from Study VI (Table 1; Fig.6B). D: Weights of treated group separated into long-term survivors (HR, closed circle) and dead (LR, closed square) compared with strain 1716 alone treated mice (Study 1, Table 1; closed triangle). Standard Error of the Mean (SEM) bars are included.

Figure 9: Detection of virus in long-term survivors by immunohistochemistry and in situ hybridization.

Long-term survivors were sacrificed and brains and other organs were fixed, sectioned and used for immunohistochemical detection of NT2 cells and HSV and in situ detection of HSV. A: The arrow identifies residual scar at tumor implantation site. B: On histology the brain shows no evidence of any tumor cells (arrow) or replicating virus. C: The residual scar site consists of dystrophic calcifications. This is a higher power view of region identified by the arrow in B. D: Latent virus was observed in the hippocompus (asterisk) of these survivors (4 months post-infection) and the insert shows the nuclear localised signal of LAT positive cells. E: MBP staining shows no evidence of demyelination in the whole brains of these mice. F: Dark-field photomicrograph of in situ hybridization performed

using a radiolabeled poly(dT) probe to detect total poly (A)$^+$RNA in cells as a measure of metabolic health of the LAT positive cells (asterisk). The experimental tissue (A serial section from 7D) was compared to uninfected, mock infected and RNAse treated tissue. There was no detectable difference in the signal in LAT positive area in adjacent serial section. (Scale Bar: =1.2mm in A, B, E; =113μm in D and -90μm in insert; =113μm in F; = 45μm in C).

EXAMPLES - SECTION 1

Materials and Methods

Animals:

Female C57B1/6 mice (4 to 6 weeks old - weight approximately 20g) were obtained from The Jackson Laboratory (Bar Harbor, ME).

Tumor Cells:

S91 Cloudman melanoma cells were obtained from the ATCC (Rockville, MD). B16, and Harding-Passey melanoma cells were a generous gift from Dorothee Herlyn (Wistar Institute, Phila, PA). Cells were grown in plastic flasks in AUTO-POW media containing penicillin, streptomycin, and 5% calf serum. When originally obtained, all cell lines were grown up, and then frozen in 95% calf serum/5% DMSO, so that all experiments could be initiated with cells of a similar passage number. On the day of intracranial injection,

cells in sub-confluent monolayer culture were passaged with 0.25% trypsin solution in EDTA, washed x1 in cell culture media, resuspended at the appropriate concentration in media without serum, and held on ice.

Intracranial tumor production:

Mice were anaesthetized with I.M. ketamine/xylazine (87 mg/kg ketamine/13 mg/kg xylazine). The head was cleansed with 70% EtOH. A small midline incision was made in the skin of the head exposing the skull. Stereotactic injection of tumor cell suspension was performed using a small animal stereotactic apparatus (Kopf Instruments, Tujunga,CA). Injections were done with a Hamilton syringe through a disposable 28g. needle. The needle was positioned at a point 2mm caudal of the bregma and 1mm left of midline. Using a separate 27g. needle with a shield that limits the length of the needle exposed to 0.5mm, the skull was breached at the appropriate coordinates. The injection needle was advanced through the hole in the skull to a depth of 2mm from the skull surface and then backed-out 0.5mm to create a potential space. $1x10^5$ cells in a total volume of 2μL were injected over 1 minute. Following the injection, the needle was left in place for 3 minutes, and then slowly withdrawn. The skin was sutured closed.

Virus:

To produce virus stocks, subconfluent monolayers of baby hamster kidney 21 clone 13 (BHK) cells were infected with HSV strains in1814, 1716, dlspTK, or wild type 17+. Virus was concentrated from the culture and titrated by plaque assay as previously described (28). All viral stocks were stored frozen in viral culture medium (AUTO-POW media containing penicillin, and streptomycin) at -70ºC, and thawed rapidly just prior to use.

Viral Inoculation:

Mice were anesthetized with I.M. ketamine/xylazine, and the head was cleansed with 70% EtOH. Using a Hamilton syringe with a 30 gauge disposable needle, the appropriate amount of virus was injected (10^4-10^6 PFU in 2μL) through a midline incision at the same stereotactic coordinates used for tumor cell injection. The injection was performed over 1 minute, and following the injection the needle was left in place for 3 minutes, and then slowly withdrawn.

Magnetic Resonance Imaging:

Mice were imaged using a 1.9 Tesla 30 cm bore animal MRI system located in the Hospital of the University of Pennsylvania MRI facility. Animals were anesthetized with I.M. ketamine/xylazine (87 mg/kg

ketamine/13 mg/kg xylazine). Subsequently, each animal was injected with 10 units of Gd(DTPA) via a tail vein. The animal was taped in place within a plexiglass gradient coil and imaged.

Immunohistochemistry:

HSV-infected cells were detected by an indirect avidin-biotin immunoperoxidase method (Vectastain ABC Kit, Vector Labs, Burlingam, CA) as specified by the manufacturer with slight modification. Briefly, tissue sections were deparaffinized, rehydrated, quenched in peroxide (H_2O_2) and blocked in 3.5% goat serum (Sigma Chem. Co., St. Louis, MO.). Tissue sections were incubated overnight at 4ºC with the primary antibody, a rabbit antiserum to HSV-1 (Dako Corp., Carpinteria, CA), used at a dilution of 1:1000. Next, the tissue was incubated at room temperature with biotinylated goat anti-rabbit IgG, the avidin-biotin horseradish peroxidase complex and finally AEC substrate. Sections were counter stained with hematoxylin and examined under the light microscope. As a control for the specificity of immuno-staining, tissues were processed as above, except that non-immune rabbit serum was substituted for the primary HSV-1 antiserum.

Titration of virus from tumor and brain:

Mice were sacrificed by lethal injection of

anesthesia. Brains with or without in situ tumors were removed aseptically, snap frozen in liquid nitrogen, and stored at -70ºC. Each tissue sample was rapidly thawed in a 37ºC water bath, and the tissue was homogenized in viral culture medium at a 10% weight/volume ratio using a Pyrex Ten Broeck tissue grinder. The homogenates were centrifuged at 3,000 x g for 10 minutes at 4ºC. The supernatant of each tissue homogenate was diluted logarithmically in media, and the viral titer of each was determined by plaque assay on BHK cells (28).

Statistics:

Standard deviation, and t-Test: two sample assuming unequal variances, were calculated using Microsoft Excel (Redmond, WA) on an apple MacIntosh computer (Cupertino, CA).

EXAMPLE 1 (lysis of melanoma cells)

In our initial studies, we wanted to make a straightforward in vitro determination of the relative abilities of HSV-1 wild type and mutant viruses to lyse various murine melanoma cells. We also wanted to compare how efficiently these melanoma cells were lysed by HSV-1 relative to baby hamster kidney (BHK) cells, which is a standard cell line used to propagate and titer HSV-1. Cells were plated in 24 well tissue culture plates at a density of $5x10^4$ cells/well. The

viruses were diluted logarithmically and cell monolayers were infected in triplicate. After 72 hours of culture, the highest dilution of virus at which complete destruction of the monolayer still occurred, was recorded for each virus-cell combination. Data are expressed as the number of PFU of virus, obtained for each virus-cell combination.

As demonstrated in Table 1, the various mutant viruses lyse melanoma cells and BHK with efficiencies similar to wild type 17+. Cloudman S-91, and H-P melanoma cells were lysed efficiently relative to BHK.

EXAMPLE 2 (tumor production)

The capacity of each melanoma cell line to produce intracranial tumors was then evaluated. For each cell line, 10 C57B1/6 mice were injected stereotactically with $5x10^4$ cells in the right cerebral hemisphere. Mice were observed daily, and sacrificed when they appeared moribund, or after 6 weeks if they remained asymptomatic. Each brain was fixed, sectioned, stained, and examined histologically for tumor. Both H-P and B-16 formed intracranial tumors in 10 of 10 C57B1/6 mice, while Cloudman S-91 only formed a tumor in 1 of 10 mice.

We decided to proceed with the H-P model, since these cells were both susceptible to lysis by the relevant HSV-1 mutants, and formed brain tumors efficiently.

Subsequent experiments verified that stereotactic injection of H-P cells into the brain of C57B1/6 mice establishes tumors in 100% of the animals. A technical advantage of this system is that the presence of a brain tumor can be verified by magnetic resonance imaging (MRI) prior to treatment, or simply by observation of a pigmented area on the skull overlying the tumor site, generally by 5 days post cell injection. The tumors progressed to a size that caused the mice to become moribund from neurologic symptoms in approximately two weeks.

EXAMPLE 3 (treatment of brain tumors with HSV-1 mutant 1716).

C57B1/6 mice were injected stereotactically in the right cerebral hemisphere with 5×10^4 Harding-Passey melanoma cells. After 10 days (Figure 1a) or 5 days (Figure 1b), 5×10^5 PFU of HSV 1716 was injected at the same stereotactic coordinates. The number of days elapsed between injection of tumor cells and time mice became moribund is shown on the X axis. Control mice were injected with an equal volume of viral culture medium at the appropriate time.

As shown in Figure 1a stereotactic injection of HSV-1 mutant 1716 into brain tumors 10 days after establishment, resulted in a statistically significant increase in the length of time elapsed until the mice become moribund ($P(T<=t)$ one-tail: 1.016×10^{-4}).

However, no long term survivors were obtained. When viral therapy was performed 5 days after tumor establishment (Figure 1b), significant improvement in outcome was again seen in the treatment group ($P(T<=t)$ one-tail: 7.707×10^{-3}), and 2/10 treated mice were cured. One long term survivor was sacrificed after day 39 post viral infection. Microscopic examination of serial sections of the brain did not reveal any residual tumor (data not shown). The second animal is still alive and asymptomatic at greater than 150 days post treatment. Treated animals that became moribund, showed progression of their brain tumors upon examination of tissue sections.

EXAMPLE 4 (1716 replication in tumor and non-tumor cells).

Immunohistochemistry shows that replication of 1716 is in fact restricted to tumor cells, and does not occur in surrounding brain. A significant number of melanoma cells within tumor were stained by polyclonal antiserum to HSV-1 on days 3 and 6 post infection. Moreover, in tumor bearing mice treated with 1716, no HSV-1 antigen staining was seen in brain tissue adjacent to tumor or in any other areas of brain in all sections examined. In addition, no histologic evidence of encephalitis was seen in any 1716 treated mice at any time. In contrast, tumor bearing mice infected with wild type 17+ virus,

exhibited multiple focal areas of HSV-1 immunohistochemical staining both within tumor and in surrounding and distant brain as well. A significant encephalitis characterised by polymorphonuclear leukocytes, nuclear dust, and extravasation of red blood cells, is seen in areas of this and other sections examined. In control experiments, no immunohistochemical staining was seen with anti-HSV-1 in tumor or brain from mice who did not receive virus, or in virally infected brain tumor sections subjected to the full immunohistochemical protocol with normal rabbit serum substituted for the primary anti-HSV-1 antibody (data not shown).

EXAMPLE 5 (kinetics of replication in tumor and non-tumor cells).

Having shown striking restriction of 1716 replication to tumor by immunohistochemistry, we next attempted to quantify the kinetics and extent of replication of 1716 in tumor by titration of infectious virus, and compare this with titration data from non-tumor bearing mouse brain infected with 1716 or 17+.

To investigate the extent of 1716 replication in brain tumors, C57B1/6 mice were injected with Harding Passey melanoma cells right of midline. Seven days later each mouse was infected with 5×10^5 PFU of 1716 at the same stereotactic coordinates. At the times

indicated, mice were sacrificed, the brains were frozen in liquid N_2 and stored at -70ºC. Specimens were thawed rapidly, homogenized, and viral titration was performed in triplicate on BHK cells (closed squares). These data represent the mean of 4 mice at each time point.

To establish the growth characteristic of 1716 and wild type 17+ in brain without tumor, mice were injected intracranially with either 5 x 10^5 PFU 1716 (open squares) or 1 x 10^3 PFU of 17+ (closed triangles). Mice were sacrificed at the times shown and processed as described above. Each point is the mean of 2 mice.

As shown in Figure 2, wild type 17+ virus replicated efficiently in non-tumor bearing mouse brain. In contrast, no replication of 1716 occurred in brain of non-tumor bearing mice. The titer of virus recovered decayed over time, and infectious 1716 could only be isolated for 3 days after inoculation. However, when 1716 was injected into brain tumors, significant replication occurred as evidenced by recovery of an amount of infectious 1716 on day 1 post inoculation that is substantially greater than the input amount. Under these conditions, infectious 1716 could be isolated from tumor bearing mice for 5 days post inoculation, but not on day 7. These results clearly demonstrate that HSV-1 mutant 1716 will freely replicate in tumor cells (leading to their

destruction) but does not replicate in non-tumor cells (leaving them unharmed).

Table 1: Relative susceptibility of melanoma cells to lysis by HSV-1.

	Cell Type		
Virus	Cloudman S91	Harding-Passey	BHK
dlspTK	10^3	10^4	$> 10^3$
1716	10^4	10^4	$- 10^3$
17+ (wild type)	10^3	10^3	$> 10^2$

EXAMPLES - SECTION 2

MATERIALS & METHODS

Virus Stocks

To produce virus stocks, subconfluent monolayers of baby hamster kidney 21 clone 13 (BHK) cells were infected with HSV strains 1716, in1814, or parental 17^+. Strain in1814 has a mutation (insertion) in the VP16 gene (located in the U_L region; Figure 3A) and strain 1716 has a mutation (deletion) in the γ34.5 gene (mutant; Figure 3C). Virus was concentrated from the culture, titered on BHK cells by plaque assay and stored at -70°C in 0.5ml aliquots of viral culture m edium (AUTO-POW media containing penicillin and streptomycin) and thawed rapidly just prior to use as described (30,34).

Culture of Tumor Cells and Differentiation of NT2

NTera-2 (clone D1) cells (referred to here as NT2 cells) were cultured as described (28,29). Briefly, the cells were passaged 1:3 twice per week in OptiMEM with 5% fetal bovine serum (FBS) and penicillin/streptomycin (P/S). The medulloblastoma cell lines, D283 MED and DAOY, were cultured in RPMI 1640 with 10% FBS, 1% P/S and 1% Glutamine. BHK cells were cultured in AUTO-POW with 5% FBS, 1% P/S and 1% Glutamine. NT2 cells were plated at a density of $2.0x10^6$ in T75 flask, and fed twice weekly with DMEM-HG supplemented with 10% FBS, 1% P/S, and 10^{-5}M retinoic acid for 5 weeks. The differentiated NT2N cells were separated from non-neuronal cells as described (29,35). On the day of intracranial injection, NT2 cells in sub-confluent monolayer culture were harvested, washed three times in buffer and placed on a bed of ice until injected into the brains of nude mice.

Plaque Assay

NT2, BHK, DAOY and D283 MED cells were plated in 24 well tissue culture plates at a density of 10^5 cells/well. The ciruses of interest were diluted logarithmically and cell monolayers were infected in triplicate with multiplicity of infections (MOI) ranging from 10 to 0.01. Cultures were observed regularly for the degree of cytopathic effects (CPE)

of the viruses and noted for each MOI.

Titration of Virus from Cell Cultures

Cells were infected at MOI=1, harvested at 4, 24 and 48 hrs post-infection and stored at -70°C. The samples were freeze-thawed twice from -70°C to 37°C, centrifuged at 3,000 x g for 10 minutes at 40°C, the supernatant was diluted logarithmically in media and the viral titer of each sample was determined by plaque assay on BHK cells (34).

Titration of Virus from Tumor and Brain

To titrate viral inoculums in tumor and brain, nude mice were intracranially inoculated with 1 x 10^6 PFU of strain 17^+ or 6.25 x 10^4 PFU of strain 1716 and the mice were sacrificed by lethal injection of anesthesia (ketamine/xylazine). The brains and tumors were dissected from mice that were sacrificed on different days post viral inoculation (day 0, 1, 3, 5 and 7), quick frozen in liquid nitrogen and stored at -70°C. The brain and tumor samples from the different time points were rapidly thawed in a 37°C water bath, and the tissue was homogenized in viral culture medium at a 10% weight/volume ratio using a Pyrex Ten Broeck tissue grinder. The homogenates were centrifuged at 3,000xg for 10 minutes at 4°C, the supernatant was diluted logarithmically in media and the viral titer of each sample was determined by plaque assay on BHK

cells (34).

Intracerebral Graft Implantation

Female homozygous nude mice (4 to 6 weeks old) were obtained from Harlan Sprague Dawley (Indianapolis, IN), the mice were anesthetized with intramuscular (IM) ketamine/xylazine (87 mg/kg ketamine/13mg/kg xylazine) and stereotactic injections of tumor cell suspensions were performed using a small animal stereotactic apparatus (Kopf instruments, Tujunga, CA), a 10μl Hamilton syringe and a 30 gauge disposable needle as previously described (35). To make cortical tumors, the syringe needle was positioned at a point 2mm rostral of the bregma and 1mm to the right of midline. The skull was cleansed with 70% ethanol and perforated with a 27 guage needle and the Hamilton syringe with the attached needle was advanced through the hole in the skull to a depth of 1.5mm below the dura and 3 x 10^4 NT2 cells in a total volume of 2μl were injected over 5 min. Prior to implantation, NT2N cells were suspended in DMEM/HG and maintained at 40°C in an ice bath. Exactly 5μl of the NT2N cell suspension, containing approximately 5 x 10^5 cells, was injected at the same location as above. Following the injection, the needle was left in place for 5 min and then slowly withdrawn over 2 min. and the superficial skin wound was closed with sutures. The mice were allowed to recover and inspected daily

for signs of illness. Body weight and cranial measurements with calipers were taken weekly. Any mice that showed signs of morbidity in extremis were sacrificed and brains were prepared for histochemistry. Tissues from some animals that died unobserved in their cage also were harvested and fixed for histochemical analysis. The experiments on nude mice are summarised in Table 2.

Viral Inoculation

Control mice and mice previously inoculated with tumor cells were anesthetized as described above and the head was cleansed with 70% ethanol. Using a Hamilton syringe with a 30 gauge disposable needle, the appropriate amount of virus was injected (10^4-10^6 PFU in 5μl) through a midline incision at the same stereotactic coordinates used for the previous injection of tumor cells. The injection was performed over 3 min following the injection and then slowly withdrawn over 1 min. Control mice received equivalent volume inoculations of viral medium.

Magnetic Resonance Imaging

The brains of selected mice were imaged using a 30 cm bore 1.9 Tesla animal Magnetic Resonance Imaging (MR1) system (General Electric). To accomplish this, mice were anesthetized as described above at various times after implantation of tumor cells and

inoculation of the tumor sites with virus. Subsequently, each animal was injected with 10 units of an enhancing agent, gadolinium complexed to a DTPA carrier (Magnevist), via a tail vein. The animal was then immobilised within a Plexiglas RF coil and imaged.

Immunohistochemical Procedures

Mice were transcardially perfused and fixed with 70% ethanol in isotonic saline (150nM NaCl,pH 7.4) or 4% paraformaldehyde (0.1M PBS.pH 7.4) and the brain as well as samples of multiple other tissues (i.e. trigeminal ganglions, heart, proximal jejunum, liver, spleen, left kidney, femur, and vertebral bodies) were dissected for histological and immunohistochemical analysis. The methods for tissue processing and light microscopic immunohistochemical analysis were similar to those described elsewhere (35,36). Both monoclonal and polyclonal antibodies to neuronal and glial cytoskeletal proteins and other polypeptides that have been shown to serve as molecular signatures of the neuronal or glial phenotype were used for the immunohistochemical characterisation of intracranial allografts (35,37). Rabbit polyclonal antisera to HSV-1 which detects the major glycoproteins present in the viral envelope and at least one core protein (Dako Corp., Carpinteria, CA) was used at a dilution of 1:1000 to detect replicating virus (38). A mouse

monoclonal antibody (MOC-1) to neural cell adhesion molecule (NCAMs) specific for human NCAMs was used at a dilution of 1:100 to detect NT2 and NT2N cells and to distinguish them from mouse brain cells (35). Another monoclonal antibody, RMO93 (1:10), which recognises rodent specific epitopes of the middle molecular weight neurofilament (NF-M) protein and does not cross-react with human NF-M was used to confirm the identity of NT2N grafts (35). RMO301 (1:100) is a monoclonal antibody that recognises human specific NF-M was used to confirm NT2N grafts. M13, a mouse monoclonal antibody which recognises human microtubule associated protein-2 (MAP2), was used at a 1:500 dilution. Rabbit polyclonal antibody specific to mouse myelin basic protein (MBP) was used at a dilution of 1:1000 (gift of A. McMorris). Tissue sections for staining with M1B-1 (a mouse monoclonal antibody that recognises a cell-cycle specific antigen (Ki-67) used at a 1:20 dilution; AMAC, Westbrook, ME) were pretreated by microwaving on 10mM Sodium Citrate as described (39). Prior to sacrifice some control mice were injected with intraperitoneally with bromodeoxyuridine (BrdU) at 5mg/g (in 150mM NaCl, 7mM NaOH) body weight in order to label NT2 cells undergoing cell division in the grafts as described (37). Segments of the proximal intestine were removed from the same mouse as positive controls for cycling cells. BrdU positive cells were identified by using

an BrdU antibody BU-33 (1:250). Antigen expressing cells were detected by the indirect avidin-biotin immunoperoxidase (Vectastain ABC kit, Vector Labs, Burlingam, CA) or peroxidase anti-peroxidase detection systems with 3,3'-diaminobenzadine (DAB) as the chromagen. Grafts and spread of virus in all animals was monitored by screening every tenth section through entire brain with MOC-1, MIB-1 and HSV antibodies.

In Situ Hybridization for HSV-1 Specific Gene Expression.

Sections of perfused and fixed tissue were mounted on slides and in situ hybridization was performed as previously described to detect viral gene expression (26,33,40). Serial tissue sections were hybridized with a ^{35}S-labeled HSV LAT specific probe (BstEII-BstEII, Fig.1E), with a ^{35}S-labeled HSV specific thymidine kinase probe (tk; an early gene product) or with a biotinylated HSV specific gC (a late gene product) probe.

Preparation of ^{35}S-labeled Nick-Translated Probe

The latency-associated transcript (LAT) probe BstEII-BstEII subfragment (0.9kb) of BamHI B was isolated from restriction digests by gel electrophoresis and purified by GeneClean (Bio 101 Inc.; La Jolla, CA; see Fig.3) (30). The 3.4kb BamHI fragment encoding the tk gene was isolated as

described (41). DNA probes were nick-translated and separated from unicorporated nucleotides by passage through Sephadex G-50 spin columns (Pharamacia) (33). The specific activities of the probes were approximately 1-5x10^8 c.p.m/μg DNA.

Preparation of ^{35}S-labeled Poly (dT) Probe

A 21-mer of poly(dT) was synthesized and was used as substrate for labeling by terminal deoxynucleotidyl transferase (TdT). Reaction mix consisted of 2μl of TdT, 1μl of Poly(dT) (6μg/μl), 5μl of 5X TdT Buffer, 6μl of $CoCl_2$ (2.5 mM), 10μl of ^{35}S-dTTP (1μg), 1μl of ddH_2O. The mix was incubated at 37°C for 30 min. and was separated from unincorporated nucleotides by passage through Sephadex G-25 spin columns. In situ hybridization was performed exactly as above except that hybridization and washes were performed at 37° in 25% formamide (42). Exposure time courses were performed on uninfected, mock infected, wild-type virus infected and RNase treated tissue sections and were used as positive and negative controls for experimental tissue sections (see 43).

Biotinylated gC Probe to Detect Active Viral Replication.

A nonisotopic in situ hybridization was performed using a 21 bp antisense gC probe (nucleic acids 199-219 of gC transcript, CGGGGCGGGGGTGGCCGGGGG; gift by

K. Montone; Fig.3F) linked to the 3′ end by a biotinylated tail [5′-$(TAG)_2$-BBB-3′]. The protocol was essentially the same as in Wang and Montone with slight modifications for mouse brain tissue.

DNA Nick End Labeling By TUNEL Method

The terminal deoxynucleotidyl transferase (TdT) dUTP-biotin nick end labeling (TUNEL) technique was performed as previously described (37). Briefly, deparaffinized and rehydrated slides were digested with 20μg/ml of proteinase K in 0.1 M Tris (pH=8) at room temperature for 15 min. After washing, the sections were incubated with a mixture containing 20mM biotinylated-dUTP, 0.3 U/μl terminal deoxynucleotidyl transferase, 1.5mM cobalt chloride, 200mM sodium cacodylate, 25mM Tris, 0.25 mg/ml bovine serum albumin (pH=6.6) at 37°C for 45 min. The reactions were stopped by washing in 2X SSC for 15 min. and the results were visualized by alkaline phosphatase conjugated with streptavidin and developed with Fast Red substrate. Coronal sections of post-natal day eight rat brain were used as positive controls for this TUNEL protocol because this was the developmental stage at which peak apoptosis activity was recognised (44).

Electron Microscopy of Thin Sections.

Portions of tumor tissues from perfused mouse

brains were fixed in 1% glutaraldehyde, 4% paraformaldehyde in 0.1M sodium cacodylate (pH=7.4) over night at 4°C and washed in sodium cacodylate buffer and processed for EM as described (37).

Statistics

Survival and weight statistics were performed using BMDP Statistics Software (ed. WJ Dixon; Release 7.0; 1993). Differences in survival in control and treated groups were compared using Generalised Wilcoxon (Breslow) Analysis. Differences in weights were compared using the t-test and the Mann-Whitney Test. Moribund animals in extremis were sacrificed and treated the same as animals found dead in their cage for statistical analysis.

EXAMPLE 6:

HSV-1 Strain 1716 Lyses and Spreads on Monolayers of Tumor Derived Human Neural Cell Lines In Vitro.

To determine how efficiently HSV-1 strain 1716 lyses rapidly dividing NT2 in comparison with parental strain 17^{+},NT2 cells were plated on 24 well plates 1 day prior to infection by these two strains at Multiplicities of Infection (MOI) of 10, 1 and to 0.1. Both viruses, at MOI of 10, lysed NT2 cells within 1 day and this was associated with the characteristic morphological changes (rounding up, phase brightness, cytomegaly, plaque formation and loss of adhesion)

associated with HSV infection. Since the behaviour of a virus at a low MOI (0.1) in vitro may predict the ability of a virus to spread in a tumor in vivo, we studied infection at MOI-0.1. Strain 1716 spread and destroyed monolayers of NT2 cells less efficiently than 17^+(1716 lysed monolayer in 3 days and 17^+ in 2 days). The behaviour of these viruses was similar in two different human medulloblastoma cell lines (D283 MED and DAOY) suggesting that strain 1716 can lyse many different brain tumor cell lines.

Next, NT2N cells (the neuron-like retinoic acid differentiated derivitatives of NT2 cells) were infected with these viruses. Strain 1716 was attenuated for cytopathicity in these cells with respect to strain 17^+ and Lactate Dehydrogenase (LDH) assays for cytotoxicity performed on NT2N cells infected with the above viruses showed that both viruses caused some non-specific toxicity within 12 hours after infection (data not shown). Interestingly, titration of virus from infected cell cultures showed that strain 1716 was deficient for replication in NT2N cells (data not shown). Because strain 1716 is more severely neuroattenuated in mice than the other engineered strains (26, 30), we conducted in vivo studies of strain 1716 versus 17^+ virus inoculated into the CNS of nude mice with and without transplants of in vitro derived NT2N cells or with tumors established from transplanted NT2 cells.

Reference is made to Table 2.

EXAMPLE 7:

Replication of HSV-1 Strain 1716 Cannot be Detected In The Mouse CNS Following Intracerebral Inoculation

Consistent with previous results using SCID mice (26), intracerebral (IC) inoculation of 5 x 10^6 plaque forming units (PFU) of strain 1716 in nude mice did not induce clinical symptoms for over 4 months post-inoculation, and there was no evidence of encephalitis on histological analysis of the brains nor any evidence of replication in the major organs (e.g. liver, spleen, bone marrow, etc.) of these mice (Table 2, Study I). In contrast to strain 1716, IC inoculation of less than 100 PFU of strain 17^+ killed nude mice within 5-10 days and histopathological anlysis revealed extensive cytopathic lesions (e.g. intranuclear inclusion bodies, cell death, etc.) in these mice (not shown).

To monitor viral replication in the brain after IC inoculation of strain 1716 versus strain 17^+, a viral titration assay was performed (Table 2, Study II). The Recovery of both viruses on day 0 was low relative to the amount of virus in the injected inoculum. This probably was due to adsorption or fusion of the viral particles to the membranes in the brain homogenates and to inactivation of virus during harvesting. Figure 4 shows that the titer of strain

17^+ exponentially increased with time and resulted in morbidity and death of inoculated mice. In contrast, the titer of strain 1716 dropped precipitously in the brains of nude mice, and virus was no longer detectable 3 days post-inoculation. Moreover, there was no immunohistochemical evidence of encephalitis in strain 1716 infected mice, and there was no detectable spread of strain 1716 virus outside the CNS as evidenced by the absence of virus in samples of liver, spleen, kidney, jejunum and bone marrow by immunohistochemistry and by in situ hybridization for HSV specific transcripts (data not shown). Likewise, direct inoculation of liver or intravenous injection with strain 1716 did not cause any morbidity or death in nude mice. In contrast to strain 1716, strain 17^+ infected mice exhibited evidence of encephalitis and tumor lysis (see Figure 4).

EXAMPLE 8:

HSV-1 Strain 1716 Lytically Replicates In NT2 Tumors But Not in Transplanted NT2N cells In The Mouse CNS

When strain 1716 was injected into NT2 tumors significant replication occurred for 7 days as evidenced by increase in viral titer over input inoculum by day 3 (Fig.4). This is in agreement with the immunohistochemical and in situ hybridization data which showed no detectable strain 1716 in the brains of these mice except in the NT2 tumor cells for as

long as they were present to support viral replication (see below).

Since quantification by titration assay in mice showed that strain 1716 replicated in NT2 cell tumors, we tested the ability of strain 1716 to induce regression of these tumors (Table 2, Study IV). To do this we injected 5 μl of strain 1716 containing 5 x 10^5 PFU into tumors that formed in the brains of nude mice following IC implantation of 3 x 10^6 NT2 cells. To monitor the fate of transplanted NT2 cells, mice were sacrificed at different time points after the viral inoculation and their brains and organs were anlysed by immunohistochemistry and in situ hybridization. NT2 cells formed tumors with a neural and epithelial histology in 100% of mice, and these tumors were lethal within 5 weeks after grafting (Fig.5A, 5B). These tumors contained abundant proliferating cells as evidenced by BrdU labeling, the immunohistochemical detection of cell cycle antigens and their rapid growth (Fig.5C, 5D). Figures 5E,F,G and H show that infection of NT2 tumors with strain 1716 is not uniform at day 3 post-infection (pi). This may reflect the localised nature of the injection site, or to cell-type specific differences in the vulnerability of the cells to infection and lysis by strain 1716. Nonetheless, most tumor cell types near the injection site harbored immunoreactive virus by day 5. At later days post-infection, more cells were infected, but

immunoreactivity for virus in the tumor was weaker presumably due to clearance of the virus following lysis of the infected tumor cells (Figure 5).

As seen in Figure 5H, viral antigen is limited to the tumors. On high magnification, the infected tumor cells showed characteristic features of HSV-1 infection, i.e. intranuclear inclusion bodies and multinuclear giant cell formation (Fig.5F). No viral antigen staining was seen in the surrounding brains of these mice or in the brains of control untreated mice. This was confirmed by a non-isotopic in situ hybridization protocol using a biotinylated probe for glycoprotein C (gC) and a radiolabeled thymidine kinase (TK) probe (a late and early gene product expressed only in acute infection) to detect active viral replication in serial sections (Fig.5J). Thus, viral replication, as evidenced by gene expression, is also restricted to the tumor cells. In contrast, tumor-bearing mice inoculated with strain 17^{+} showed viral replication in both tumor and brain (Fig.5K, 5L). Mouse brains harvested 18 days after viral treatment of their tumor implants with 1716 showed a marked decrease in the size of tumor. Indeed only a residual fibrotic scar was seen in some mice, and viral antigen was strictly limited to the remaining cells in the scar (Fig.5 M, N, O).

To examine the ability of strain 1716 to induce regression of brain tumors, NT2 tumors in the brains

of nude mice were stereotactically injected with strain 1716 twelve days after implantation of NT2 cells. Treated mice were inoculated with 5 x 10^5 PFU (in 5μl) of strain 1716 at the tumor implantation site and control mice received 5μl medium alone. MRI scans showed that these mice developed detectable tumors by 11 days post-implantation. In all mock treated tumor-bearing mice, the tumor grew rapidly in size and was lethan (Fig.6A-D). In all treated mice, strain 1716 infection induced a detectable regression of the tumor at the original inoculation site (Fig.6E-G).

To determine whether transplanted NT2N cells were permissive for HSV replication, 2.5x10^5 cells were transplanted into the brain parenchyma or ventricles of nude mice (Table 1; Study III). These cells integrate and survive for over a year and acquire a fully mature post-natal human CNS neuronal phenotype (27). Strain 1716 was then inoculated at the same stereotactic site 6 weeks post-implantation. The grafts were identified and distinguished from mouse cells by using human specific (MOC1 and RMO301) and mouse specific (RM093) antibodies to neural cell adhesion molecule (MOC1) or neurofilament proteins (RM0301, RM093). In contrast to NT2 tumors, these long-term NT2N transplants were non-permissive for strain 1716 replication as evidenced by lack of immunohistochemical staining for viral antigens at days 1,3,5,7,9,21 and 50 post-viral inoculation.

EXAMPLE 9:

HSV Strain 1716 Induces A Non-Apoptotic Death In NT2 Tumor Cells.

Sections of tumor from a selected group of strain 1716 infected mice and uninfected tumor controls were prepared for EM analysis to characterise the mode of cell death in the NT2 cell tumors. The infected cells on H&E staining had the characteristics typical of HSV infected cells (Fig.5F). Viral assembly domains can be seen in the nucelus of infected cells. There was no evidence for an apoptotic mechanism of cell death in the virally infected tumor cells by EM (Fig.7). Tumor cells with viral particles showed the fragmentation and dissolution of nuclei and organelles as well as condensed and marginated DNA. Terminal deoxynucleotidyl transferase (TdT)dUTP-biotin nick end labeling (TUNEL) and DNA gel electrophoresis studies did not show evidence of DNA fragmentation indicative of apoptosis (data not shown). Taken together, these findings indicate that strain 1716 induces the characteristic lytic infection in the NT2 cells in vitro and in vivo.

EXAMPLE 10:

Long-term Survival of HSV Strain 1716 Treated Tumor-Bearing Mice.

Based on the results of the studies described above, we analysed the survival of a cohort of tumor-bearing

mice that were or were not treated with virus (Table 2, Studies V and VI). This also enabled us to assess the long-term consequences of treatment with strain 1716 (see below). Twenty mice were inoculated with $3x10^6$ NT2 cells and several days later mice were split into two groups. Control mice received culture medium and treated mice received $5x10^6$ PFU of strain 1716. In the first survival experiment (Table 2, Study V) virus treatment was given at 12 days post-tumor cell inoculation. There was only 1 (10%) long-term survivor in this group and there was no significant difference in survival between control (Study VA) and treated (Study VB) animals (p=.63, Fig.8A). Histological examination of some control and treated animals showed that virus was replicating in the tumor. In the treated animals it appears that the tumor had already grown and spread to such a size that one virus treatment was not sufficient to induce regression of the tumor (data not shown). When virus treatment was given at 7 days post-tumor cell inoculation, 44% of the mice survived long-term and 100% of control mice died within 10 weeks (Table 2, Study VI; Fig.6B). This resulted in a statistically significant improvement in survival (p<.03;median survival time (controls)=44.75+/-5.24: median survival time (treated)= 101.78+/-22.69).

In the survival experiments, significant weight loss was observed in a sub-population of both mock-

treated tumor-bearing mice (Table 2, Study VIA) and strain 1716 treated tumor-bearing mice (Table 2, Study VIB). Since there was no difference in the average weights of these two groups (Fig.8C) we separated the treated group (Study VIB) into two sub-groups (High-Responders (HR)-the long-term survivors (4 mice) and Low-Responders (LR)- mice that died (5 mice); Fig 8C). We found a significant difference in weight loss at 6 weeks ($p<0.01$) between groups HR and LR and between groups LR and 1716 treated mice (Study 1) using the standard t-test (Fig.8D). These data confirmed earlier observations of weight loss in Study V, and they suggest that the weight loss may be due to the toxic physiological effects of tumor growth, regression or lysis and not directly due to an effect of the viral infection of the brain. Notably, some of the treated mice that died showed leakage of tumor cells from implantation site into ventricles and leptomeninges. Since this would lead to obstruction of the flow of cerebrospinal fluid, it is not surprising that some of these treated mice had survival kinetics similar to control mice (Fig.8A). Finally, intracranial volume of the control mice with brain tumors increased by over 25% (indicating tumor growth) while in treated mice intracranial volume did not increase significantly (see Fig.6). The long-term survivors also did not have a significant increase in intracranial volume (data not shown). These

interpretations are supported by the fact that mice inoculated with strain 1716 alone did not show any clinical or histological characteristics of encephalitis at any time during the study

Long-term survivors from studies V and VI (Table 2) had no clinical symptoms, no atypical increase in intracranial volume, and no weight loss (Fig.8C). These mice and mice inoculated with strain 1716 alone were sacrificed and analysed for pathology and viral replication by immunohistochemistry and in situ hybridisation. In the survivors, there was only evidence of fibrotic scar tissue and dystrophic calcifications but no evidence of residual, live tumor cells (Fig.9A,B,C). Immunohistochemical staining for cell cycle antigens (e.g. using MIB-1) was also negative suggesting the absence of any cycling NT2 cells in the brain. Further, the brains of these mice were negative for herpes antigens indicating the absence of replicating virus, although in some mice in situ hybridization revealed the presence of latent HSV in the hippocampus (Fig.9D). Surprisingly, latent virus was also found in the hippocampus of mice infected with strain 1716 along. However, examination of representative rostral to caudal levels of the brains of all survivors using antibodies specific to HSV and to the human NT2 cells (MOC-1, RMO301), did not reveal any evidence of active viral replication nor any residual live tumor cells. To exclude the

possible occurrence of other potential toxic sequelae of HSV-1 strains such as demyelination, we probed sections from the brains of mice using antibodies to myelin basic protein. Examination of mice injected with strain 1716 alone as well as long-term survivors revealed no evidence of demyelination (Fig.9E). Finally, we monitored the levels of poly(A)$^+$RNA by in situ hybridisation using a radiolabeled poly(dT) probe to assess the overall metabolic health of neurons (31,32), and we found no quantifiable difference in the level of poly (A)$^+$RNA between the LAT-positive cells of the long-term survivors versus the contralateral non-LAT positive cells in the same mice and in uninfected, control mice (Fig.9F).

TABLE 2: Summary of Animal Experiments

Study	Treatment			Survival	Clinical Disease
	Tumor	Virus (p.i.[a])	Number		
I.	-	1716	4	100% (>16wks)	n.a.
II.A	-	17^+	10	n.a.[a]	Encephalitis
B	-	1716	10	n.a.	n.a.
C	NT2	1716(12)	10	n.a.	n.a.
III.	NT2N	1716(6wks)	10	n.a.	None
IV.A	NT2	-	10	b	None
B	NT2	1716(14)	10	b	None
C	NT2	17^+(14)	2	b	Encephalitis
V.A	NT2	-	10	0%(<8wks)	Cachexia[c]
B	NT2	1716(12)	10	10%(>35wks)[e]	None[d]
VI.A	NT2	-	8	0%(<10wks)	Cachexia[c]
B	NT2	1716(7)	9	44%(>25wks)[f]	None[d]

[a]p.i.-post cell implantation when virus or mock inoculum was administered. n.a. - not applicable.
[b]mice were sacrificed at different days after viral inoculation to follow the kinetics of tumor progression and viral spread (see text for details).
[c]These mice also showed no symptoms but the mice that died showed the same range of symptoms as in mock treated mice.
[d]Long-term survivors showed no symptoms but the mice that died showed same range of symptoms as in mock treated mice.
[e]$p=0.63$, no statistically significant difference in survival between mock and treated mice.
[f]$p<0.03$, statistically significant difference in survival between mock and treated mice.

CONCLUSION

HSV type I (HSV-1) strain 1716 has a deletion in the γ34.5 neurovirulence gene which renders it avirulent in the mouse CNS, we have assessed its potential to induce selective lysis of tumor cells versus neurons in vitro and in vivo. Parental HSV-1 strain 17^+ and engineered strain 1716 were studied using human teratocarcinoma derived embryonal carcinoma cells (NT2 cells). These cells resemble neuronal progenitor cells and can be induced to differentiate into neurons (NT2N) cells) with retinoic acid. Intracerebral grafts of NT2 cells into the brains of nude mice resulted in lethal brain tumors while grafts of NT2N cells resulted in the integration and maturation of NT2N cells without neoplastic reversion. In vitro studies showed that strain 1716 replicates in and spreads on monolayers of NT2 cells, resulting in the lysis of these cells. However, strain 1716 did not replicate in NT2N cells in vitro. In vivo, strain 1716 replicated preferentially in NT2 tumors as evidenced by immunohistochemical staining for viral antigens, in situ hybridisation for HSV specific transcripts and by titration of virus from brains with tumor following intracranial injection of the virus into these mice. In contrast to NT2 tumor cells, transplanted NT2N cells were non-permissive for strain 1716 replication. The temporal regression of NT2 tumors in mice treated with strain 1716 was

demonstrated in vivo by Magnetic Resonance Imaging. Electron microscopy and studies of DNA fragmentation suggested that regression of NT2 brain tumors in strain 1716 treated mice was mainly due to a non-apoptotic, lytic mode of cell death. Strain 1716 treated NT2 tumor-bearing mice survived over twice as long as mock-treated tumor bearing mice and these differences in survival (25 vs.9 wks.) were statistically significant ($p<.03$). We conclude from these studies that strain 1716, a replication-competent, non-neurovirulent mutant of HSV-1, induces regression of human neural tumors established in the brains of nude mice resulting in their prolonged survival. These results indicate that HSV-1 γ34.5 mutants are candidates for the treatment of human brain tumors in vivo.

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CLAIMS

1. Use as an anti-cancer agent of a mutant herpes simplex virus wherein the mutant virus comprises a modification in the γ34.5 gene in the long repeat region (R_L) such that the γ34.5 gene is non-functional.

2. Use of a mutant herpes simplex virus according to claim 1 wherein the virus is substantially non-neurovirulent.

3. Use of a mutant herpes simplex virus according to claim 1 or claim 2 wherein the mutant virus is selected from a mutant HSV-1, HSV-2 or derivatives thereof.

4. Use of a mutant herpes simplex virus according to any preceding claim wherein the modification to the virus is made within the Bam H1 s restriction fragment of the R_L terminal repeat.

5. Use of a mutant herpes simplex virus according to claim 4 wherein the modification is a deletion of from 0.1 to 3kb, in particular of from 0.7 to 2.5 kb.

6. Use of a mutant herpes simplex virus according to claim 5 wherein the deletion is a 759 bp deletion in the γ34.5 gene.

7. Use of a mutant herpes simplex virus according to any of the preceding claims as an anti-brain tumour agent.

8. Use of a mutant herpes simplex virus according to claim 7 against primary tumours originating within the brain and nervous system.

9. Use of a mutant herpes simplex virus according to claim 7 against metastatic tumours, in particular against metastases of melanoma cancers.

10. Use of a mutant herpes simplex virus according to any of the preceding claims wherein the use is in a mammal, in particular in a human.

11. Use according to any of the preceding claims wherein the mutant herpes simplex virus is mutant 1716.

12. Use of a mutant herpes simplex virus according to any preceding claim in the manufacture of a medicament for the treatment of cancer in mammals, in particular in humans.

13. Use of a mutant herpes simplex virus according to claim 12 in the manufacture of a medicament for the treatment of brain tumours in mammals, in particular

in humans.

14. Use of a mutant herpes simplex virus according to claim 12 or claim 13 in the manufacture of a medicament for the treatment of primary tumours originating within the brain and/or nervous system.

15. Use of a mutant herpes simplex virus according to any of claims 12 to 14 in the manufacture of a medicament for the treatment of metastatic tumours, in particular against metastases of melanoma cancers.

16. A method of treating cancer in mammals, in particular in humans by administering a pharmaceutical formulation comprising a mutant herpes simplex virus wherein the mutant virus comprises a modification in the γ34.5 gene in the long repeat region (R_L) such that the γ34.5 gene is non-functional.

17. A method of treating cancer in mammals, in particular in humans according to claim 16 by administering a pharmaceutical formulation directly into the tumour.

18. A method of treating cancer in mammals, in particular in humans according to claim 16 by administering a pharmaceutical formulation parenterally into the blood stream feeding the tumour.

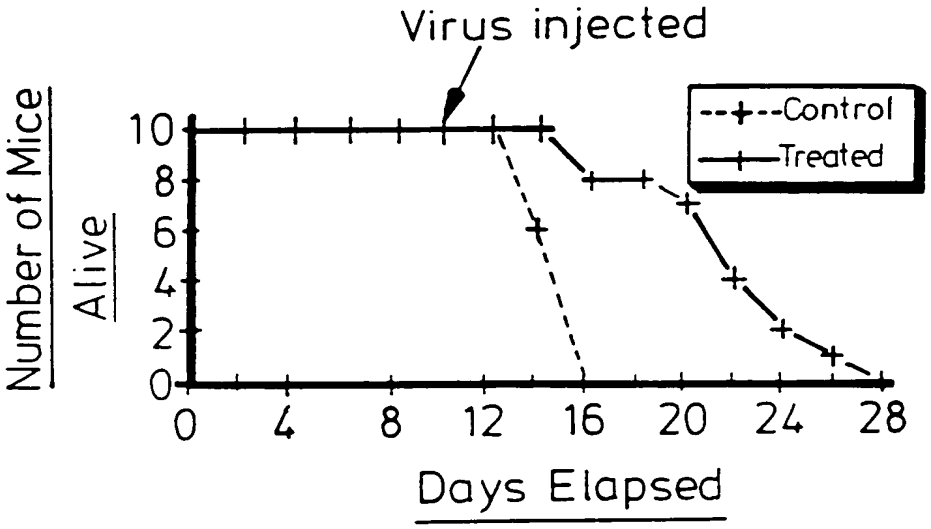
Virus injected
Number of Mice Alive
10
8
6
4
2
0
0 4 8 12 16 20 24 28
Days Elapsed
--+--Control
—+—Treated

FIG. 1a

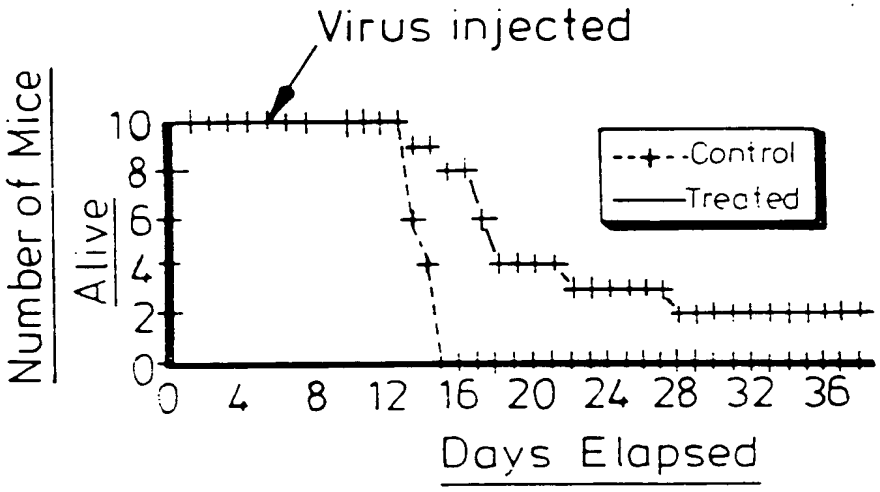
Virus injected
Number of Mice Alive
10
8
6
4
2
0
0 4 8 12 16 20 24 28 32 36
Days Elapsed
--+--Control
——Treated

FIG. 1b

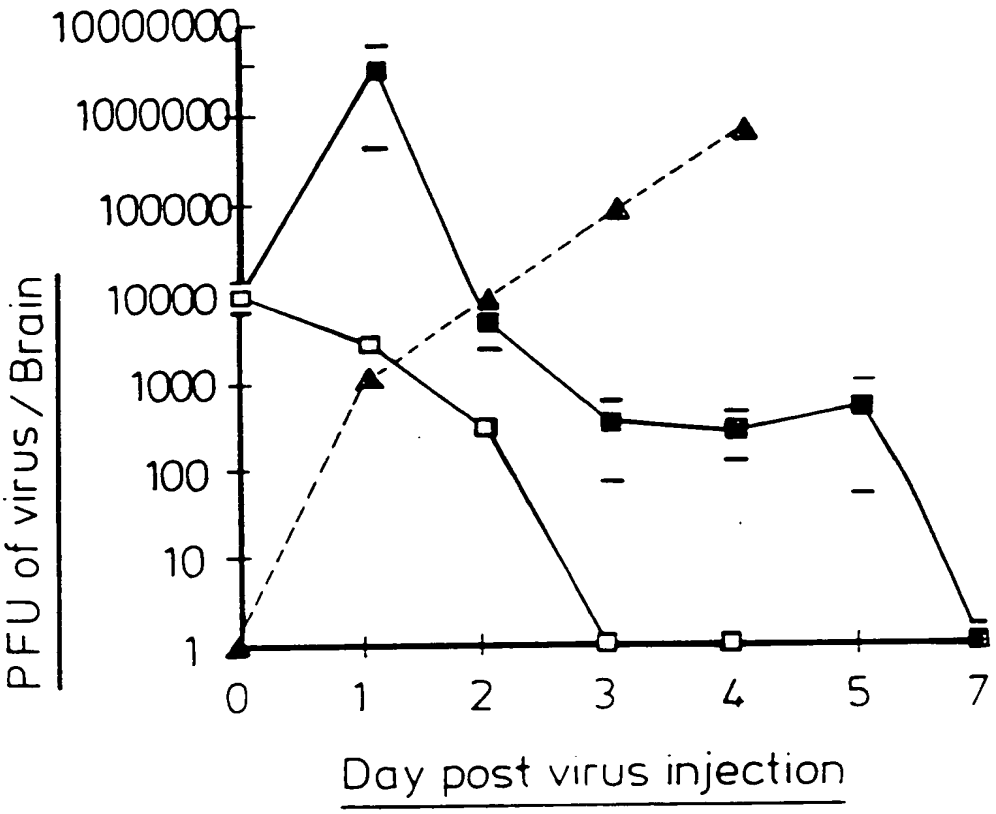
10000000
1000000
100000
10000
1000
100
10
1
PFU of virus / Brain
0
1
2
3
4
5
7
Day post virus injection

FIG. 2

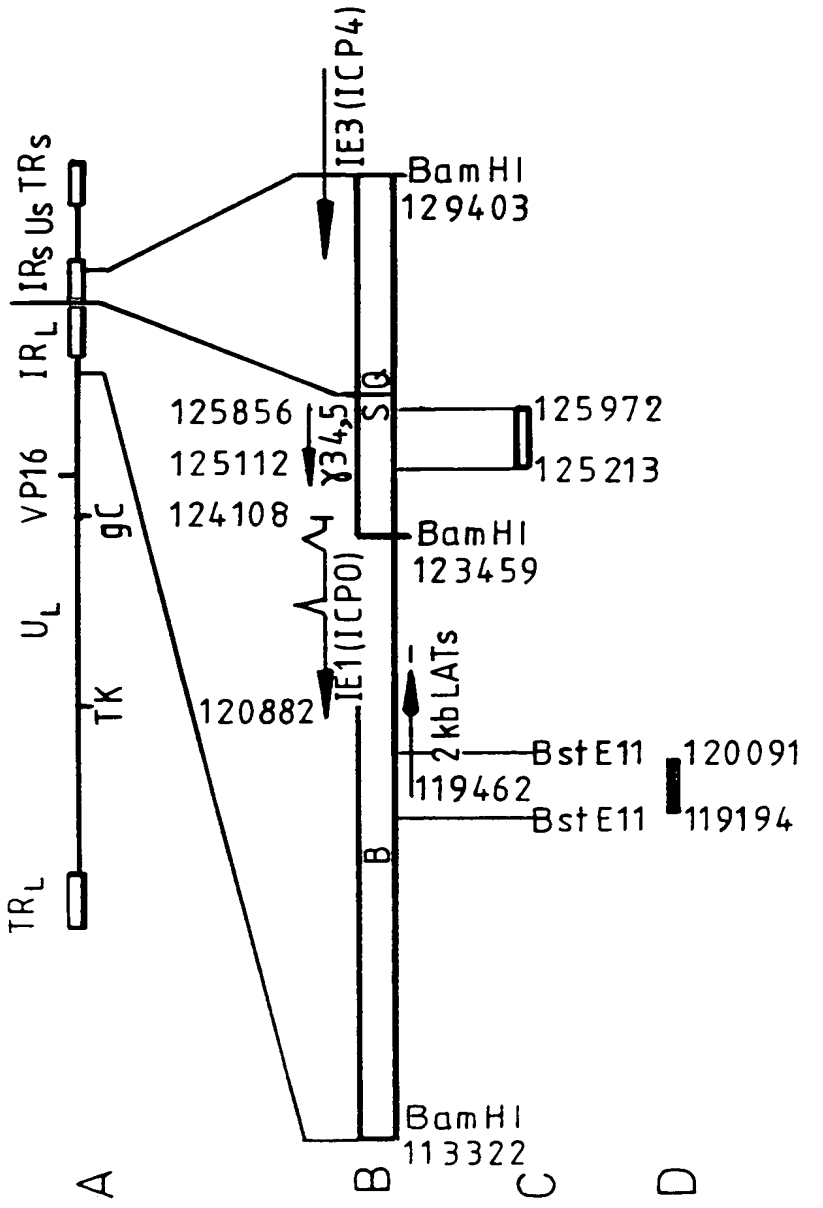
IE3(ICP4)
BamHI
129403
IRs Us TRs
IRL
125856
γ34,5
125112
124108
125972
125213
BamHI
123459
VP16
gC
UL
IE1(ICP0)
2kb LATs
120882
BstE11
120091
119462
BstE11
119194
TK
TRL
BamHI
113322
A
B
C
D

FIG. 3

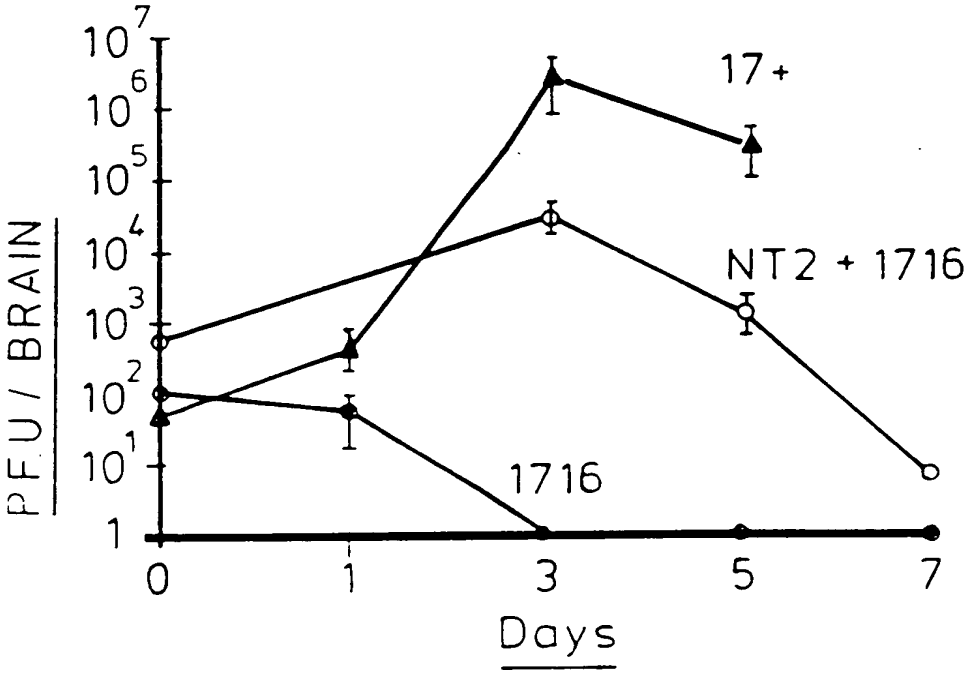
PFU / BRAIN
10^7
10^6
10^5
10^4
10^3
10^2
10^1
1
17+
NT2 + 1716
1716
0
1
3
5
7
Days

FIG. 4

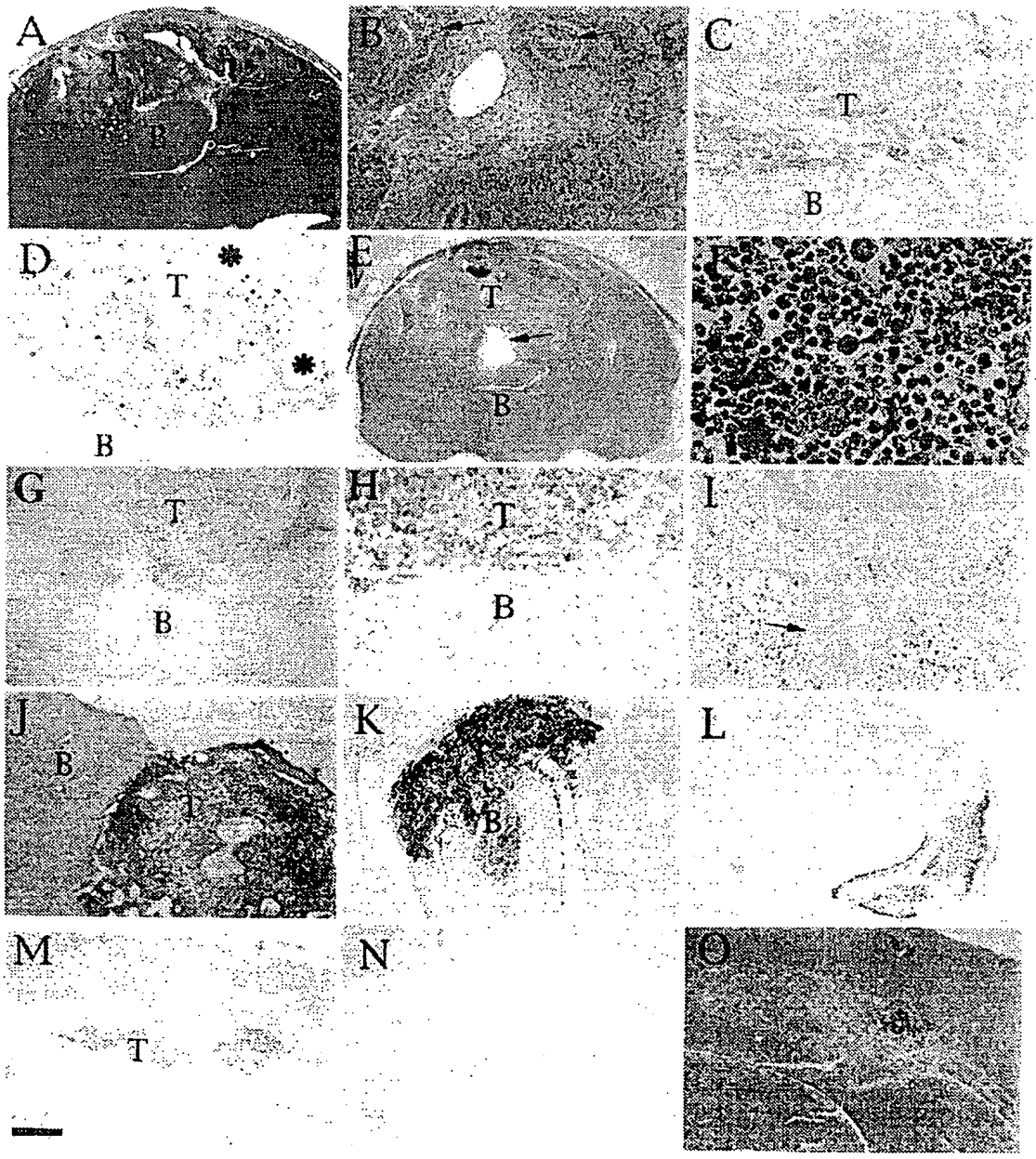

FIG. 5

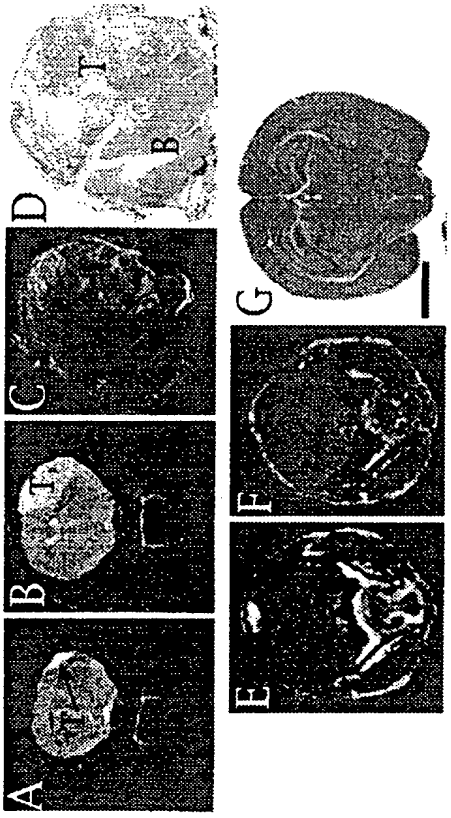

FIG.6

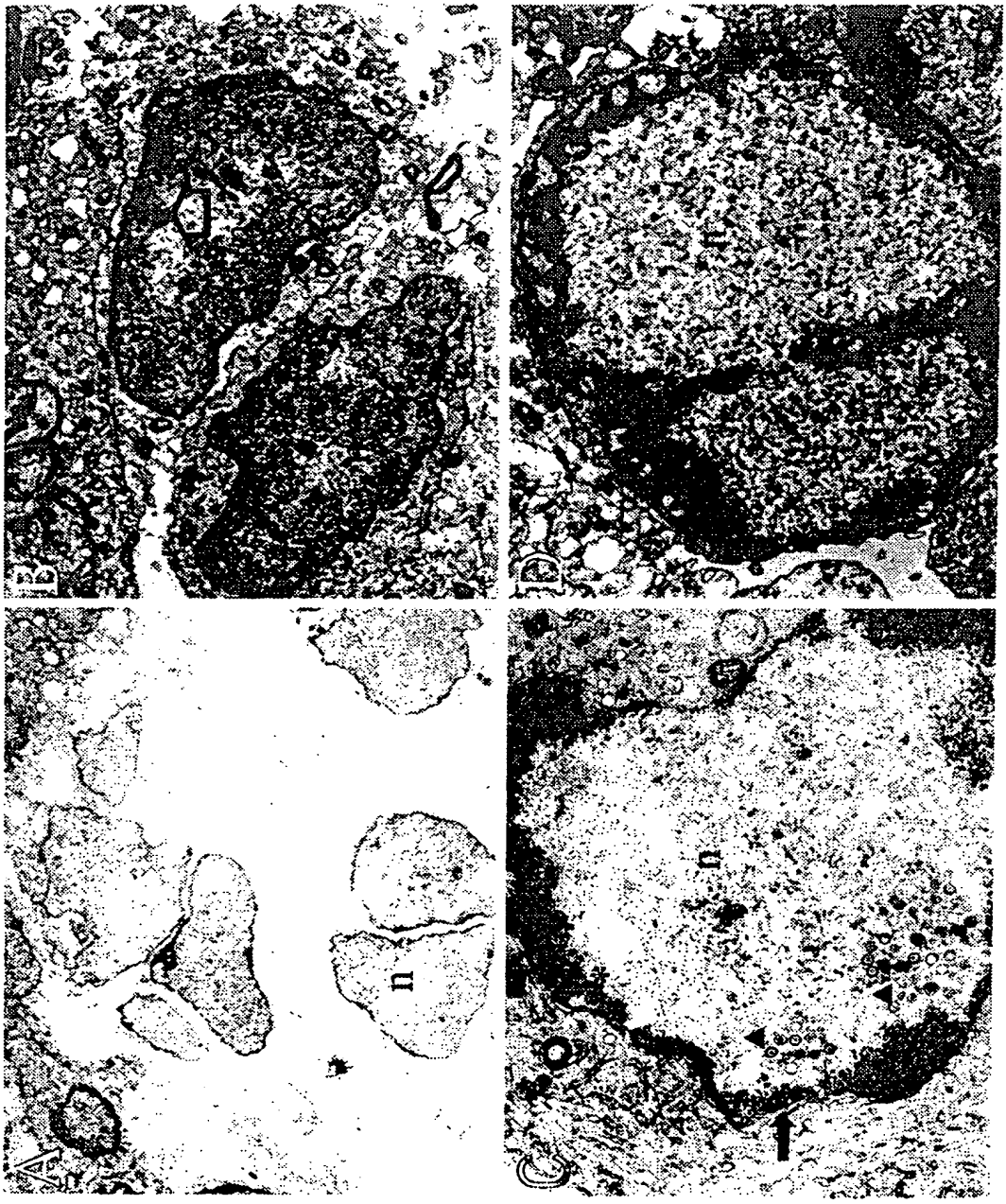

FIG. 7

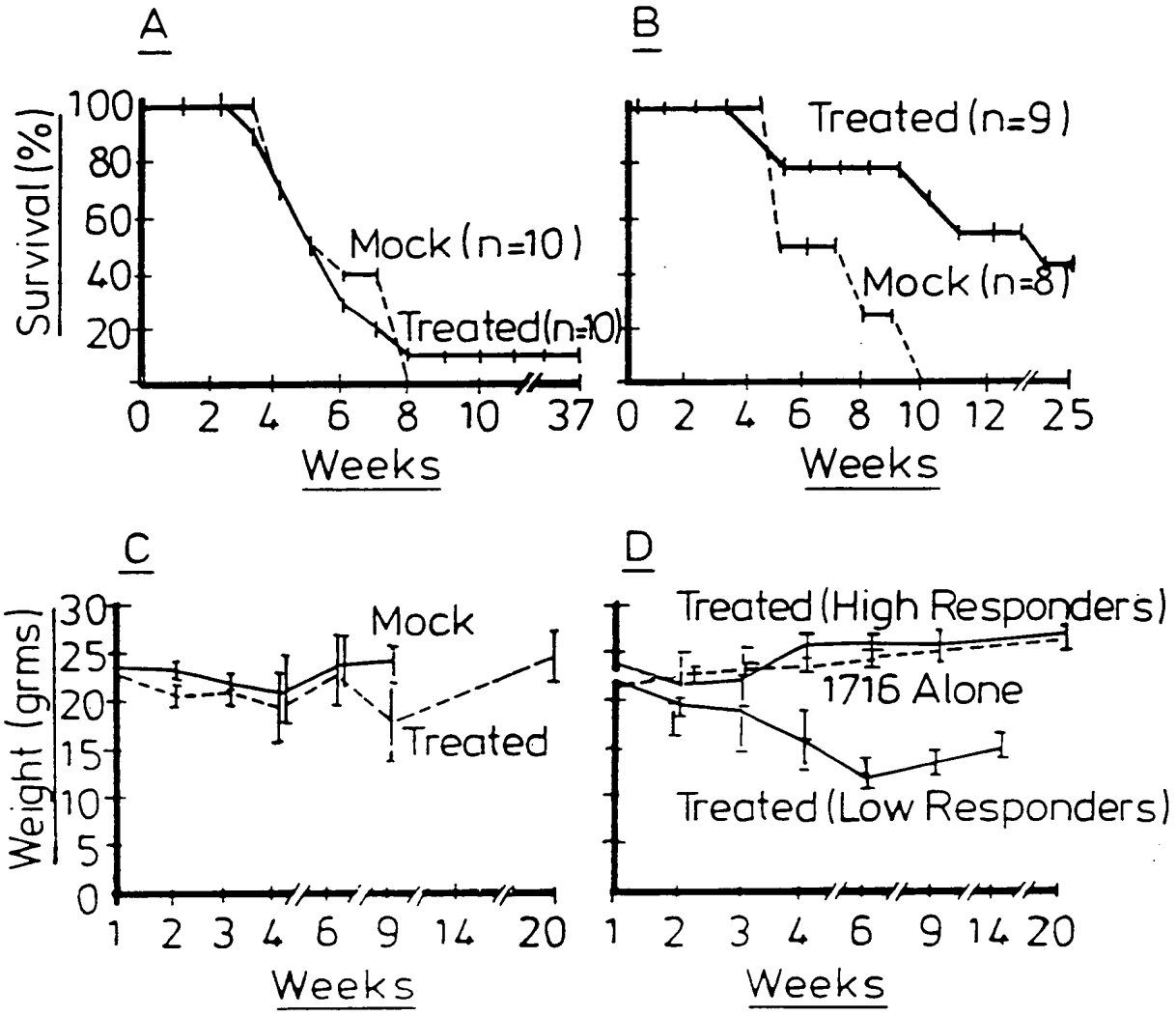
A
B
Survival (%)
100
80
60
40
20
Mock (n=10)
Treated (n=10)
0 2 4 6 8 10 37
Weeks
Treated (n=9)
Mock (n=8)
0 2 4 6 8 10 12 25
Weeks
C
D
Weight (grms)
30
25
20
15
10
5
0
Mock
Treated
1 2 3 4 6 9 14 20
Weeks
Treated (High Responders)
1716 Alone
Treated (Low Responders)
1 2 3 4 6 9 14 20
Weeks

FIG. 8

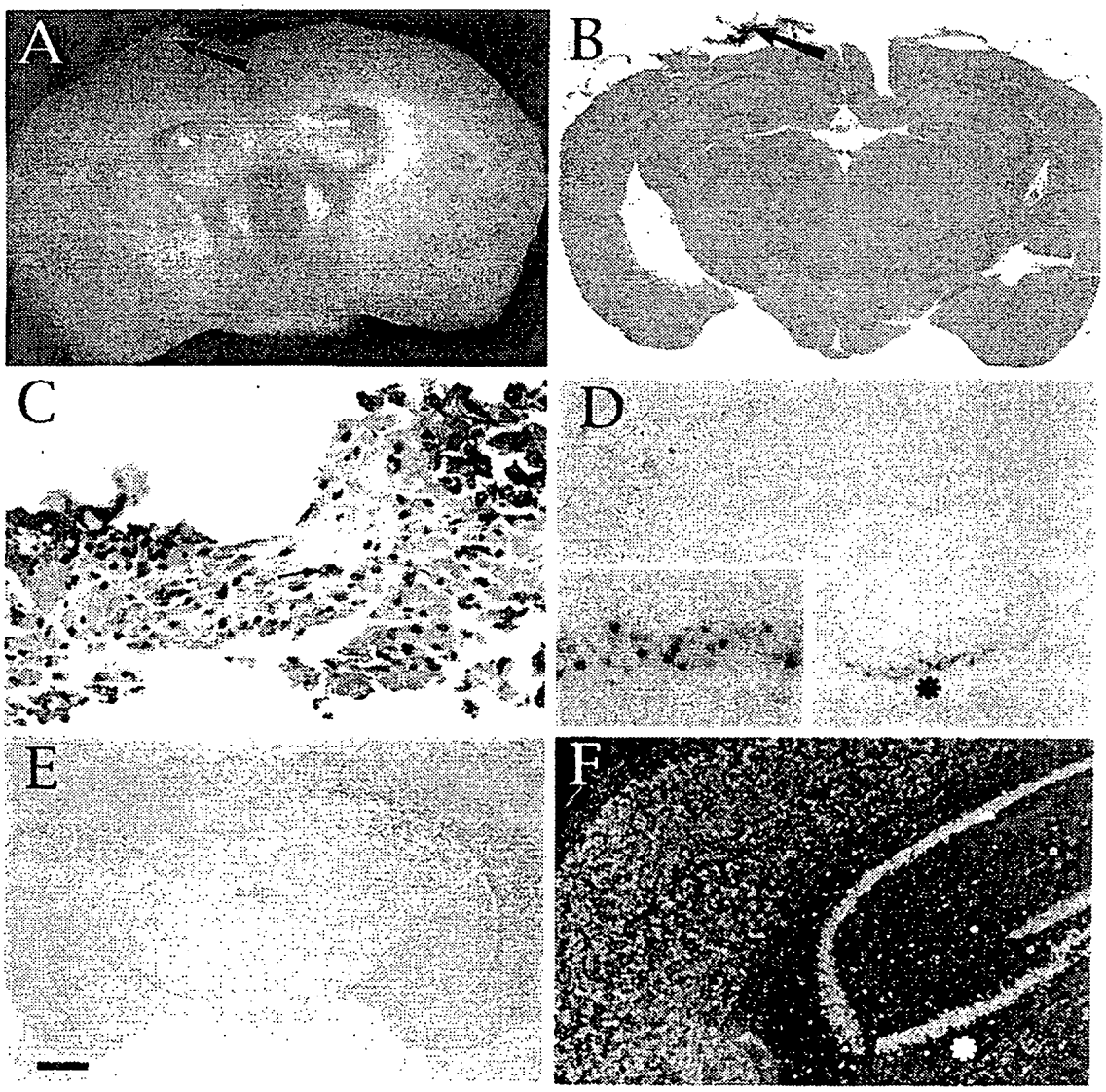

FIG. 9

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1 Please give the title of the invention

CANCER TREATMENT

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Agent's address 19 Royal Exchange Square
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CANCER TREATMENT

The present invention relates to the use of a herpes simplex virus (HSV) mutant for the treatment of cancer tumors, particularly those of the brain or nervous system whether the tumors are metastatic tumors or primary tumors.

The DNA sequence of herpes simplex type 1 (HSV-1) is known (references 14,33) and is linear with a length of about 152k residues. It consists of two covalently linked segments, designated long (L) and short (S). Each segment contains a unique sequence flanked by a pair of inverted terminal repeat sequences. The long repeat (R_L) and short repeat (R_S) are distinct. The unique long (U_L) region includes genes UL1 to UL56, and the U_S region includes genes US1 to US12.

A relatively large number of patients with advanced cancers will develop metastatic lesions in the brain and spinal cord. This frequently results in severe and debilitating neurological complications including headache, paralysis, seizures, and impaired cognition. It has been estimated that 70,000 cancer deaths occur each year in the United States with metastatic lesions to the central nervous system (CNS) (1). Radiation and steroids are presently the principle therapies used, however, they are only palliative, and frequently cause significant neuropsychological and endocrinological morbidity (1).

Surgery is generally reserved for removal of solitary metastases, and is often not curative (2).

Viral therapy for the destruction of tumors is not a new concept. Effects in various experimental tumor systems have been demonstrated using parvovirus H-1, Newcastle disease virus, retroviral vectors containing drug susceptibility genes, and Herpes Simplex Type I virus (HSV-1) (3-8). The mechanisms by which viruses improve the outcome in experimental tumor systems are complex and poorly understood. Brain tumors represent a dividing cell population occurring within an essentially non-dividing cell population of support cells, and terminally differentiated neurons. Thus, in the context of brain tumor therapy, one rationale is to select a virus that replicates exclusively or preferentially in dividing cells. Such a virus may be capable of establishing a lytic infection exclusively in tumor cells within the CNS, ultimately destroying the tumors without infecting surrounding brain, and without deleterious effects to the host.

Pioneering experiments with HSV showed a dose dependent improvement in survival of nude mice bearing intracranial human gliomas following intratumoral therapy with mutant HSV-1 dlspTK (4). This virus has a deletion in the viral thymidine kinase (TK) gene, (9) and exhibits a relatively neuro-attenuated phenotype in mice (10). However, dlspTK infection of

tumor bearing animals causes histologically evident encephalitis (4). The use of TK$^-$ mutants of HSV-1 for viral therapy also has an inherent major disadvantage in that these viruses are resistant to the clinically effective anti-viral agents acyclovir and ganciclovir (11).

The terminal 1 kb of the long repeat region (R_L) of the HSV-1 and HSV-2 genomes contain a gene (12-14), that confers neurovirulence. Deletion or mutation of this gene (γ34.5), results in variants that grow as well as wild type virus on dividing cells of many established cell lines, but show impaired replication on non-dividing cells (13-15). In mice, γ34.5 null mutants are incapable of replicating in the central nervous system, and do not cause enceplhalitis (13,16-18).

A mutant HSV-1 called R3616, containing a 1000 base pair (bp) deletion in γ34.5, with an LD_{50} (minimum dose of virus that kills 50% of infected animals) that is at least $3x10^3$ fold greater than wild type F strain virus from which it was derived (13), has been shown to improve the outcome of nude mice bearing intracranial human gliomas (19). In the work presented here, we have utilized an HSV-1 strain 17 mutant virus called 1716, that has a 759 bp deletion in γ34.5 (18).

The construction of mutant virus 1716 is described in published patent application WO92/13943

(PCT/GB92/00179) the contents of which are incorporated herein by reference. However, this patent publication is solely concerned with the use of mutant 1716 as a vaccine, either in itself or as a vector vaccine which includes a heterologous gene coding for an antigen.

Melanoma is a prevalent malignancy. Cerebral metastases occur in up to 75% of patients with metastatic disease, and are among the most common causes of death (20-24). Presently, the life span of patients with CNS melanoma is short, ranging from 2 to 7 months (25).

It is an object of the present invention to provide an improved HSV-based viral therapy of cancer tumors.

The present invention in one aspect provides the use as an anticancer agent of a mutant herpes simplex virus which has been modified in the γ34.5 gene of the long repeat region (R_L) such that the gene is non-functional.

The invention also relates to a method of treatment of cancer in a mammal (human or animal) by the administration to the mammal of an anti-cancer effective dose of the mutant herpes simplex virus.

By "non-functional" we mean that the gene has been modified by deletion, insertion or substitution (or other change in the DNA sequence such as by rearrangement) such that it does not express the

normal product or a functionally equivalent product. The effect of the non-functionality of the gene is that the neurovirulence of the virus to the patient is substantially removed.

Thus the invention is based on the finding that rendering the γ34.5 gene non-functional provides an HSV mutant which is particularly effective in destroying dividing tumor cells, whilst at the same time the HSV mutant does not replicate within normal non-cancerous cells. It therefore has the potential to provide a safe anti-cancer treatment.

Two types of herpes simplex virus are known HSV-1 and HSV-2 and either may be employed in the present invention to provide the HSV mutant. Intertype recombinants containing DNA from both types could also be used.

The modification may be effected at any convenient point within the γ34.5 gene, and such point generally corresponds to a restriction enzyme site. The modification may be within the Bam H1 s restriction fragment of the R_L terminal repeat (corresponding to 0-0.02 and 0.81 - 0.83 mu). The modification is typically a deletion of 0.1 to 3kb, particularly 0.7 to 2.5kb. In this work we employed a 759 bp deletion in γ34.5 in the HSV-1 mutant referred to as 1716.

The HSV genome also includes a number of other genes which are non-essential to the successful

culturing of the virus. It is, of course, necessary to retain within the HSV mutant the ability to culture the mutant so that the mutant is self-replicating and stocks of the mutant can be grown in tissue culture. Lethal modifications of the genome which remove the ability to culture the HSV mutant are not acceptable.

However, in addition to the primary modification to the γ34.5 gene of the R_L region, it may be advantageous to also include in the HSV mutant one or more secondary non-lethal modifications within non-essential genes. Again these modifications may be by way of deletion, insertion or substitution etc. so as to render the gene non-functional. Patent specification WO91/02788 discloses an HSV-1 mutant which includes a modification within the UL48 gene coding for Vmw65 which removes the transinducing properties of the Vmw65 transactivator protein. The herpes virus is able to remain in a latent state within a patient, in which state the virus is present but is not actively replicating. Deactivation of the Vmw65 transactivator protein maintains the virus in its latent state. The HSV mutant of the present invention may thus also include a secondary genetic modification, such as a modification within the Vmw65 gene (referred to as mutant 1764).

The present invention also encompasses as a new product an HSV mutant which includes in addition to the primary modification, a secondary non-lethal

modification (for example within Vmw65). The mutant may be derived from HSV-1 or HSV-2.

In a similar way, other secondary modifications may involve modification of the latency associated transcript (LAT) promoter so as to render the promoter non-functional and prevent transcription thereof.

Herpes simplex virus infects the brain and nervous system. The HSV mutant is effective against primary tumors originating within the brain and nervous system, but is particularly useful against metastatic tumors where cancer cells originating elsewhere have lodged in the brain or nervous system (particularly the central nervous system (NS)). Brain metastases occur commonly in a variety of human cancers (e.g. melanomas),and at present such cases are invariably fatal. The efficacy of treatment according to the invention employing the HSV mutant will depend on the time after origination of the tumor at which the treatment is initiated, but efficacy is improved by early treatment for example in 1 to 30 days.

The LD_{50} (minimum dose of virus that kills 50% of infected animals) of the 1716 mutant in respect of mice is 10^6 fold greater than that of the wild type 17+ virus from which it is derived. Thus the neurovirulence of 1716 is essentially removed relative to the wild type virus.

The effective non-toxic dose of HSV mutant can be determined by routine investigation by the skilled

man, and will depend on a number of factors including the particular species of mammal and the extent of development of the tumor. A guide can be obtained from the Examples herein.

The HSV mutant will generally be administered by injection either directly into the tumor or adjacent thereto, or parenterally into the blood stream feeding the tumor.

It will usually be presented as a pharmaceutical formulation including a carrier or excipient, for example an injectable carrier such as saline or apyrogenic water. The formulation may be prepared by conventional means.

Embodiments of the invention will now be described by way of example only.

Figures 1 and 2 show the results of experiments described fully in Example 3 and 5 respectively.

Figure 1 shows survival curves for tumor-bearing mice injected at 10 days (Figure 1b) and at 5 days (Figure 2b) post tumor injection with HSV-1 mutant 1716; and

Figure 2 shows the relative replication rates of HSV-1 mutant 1716 in brain tumor (closed squares); and of 1716 and wild type 17+ in non-tumor brain (open squares and closed triangles respectively).

EXAMPLES

Materials and Methods

Animals:

Female C57B1/6 mice (4 to 6 weeks old - weight approximately 20g) were obtained from The Jackson Laboratory (Bar Harbor, ME).

Tumor Cells:

S91 Cloudman melanoma cells were obtained from the ATCC (Rockville, MD). B16, and Harding-Passey melanoma cells were a generous gift from Dorothee Herlyn (Wistar Institute, Phila, PA). Cells were grown in plastic flasks in AUTO-POW media containing penicillin, streptomycin, and 5% calf serum. When originally obtained, all cell lines were grown up, and then frozen in 95% calf serum/5% DMSO, so that all experiments could be initiated with cells of a similar passage number. On the day of intracranial injection, cells in sub-confluent monolayer culture were passaged with 0.25% trypsin solution in EDTA, washed x1 in cell culture media, resuspended at the appropriate concentration in media without serum, and held on ice.

Intracranial tumor production:

Mice were anesthetized with I.M. ketamine/xylazine (87 mg/kg ketamine/13 mg/kg xylazine). The head was cleansed with 70% EtOH. A small midline incision was made in the skin of the head exposing the skull. Stereotactic injection of

tumor cell suspension was performed using a small animal stereotactic apparatus (Kopf Instruments, Tujunga,CA). Injections were done with a Hamilton syringe through a disposable 28g needle. The needle was positioned at a point 2mm caudal of the bregma and 1mm left of midline. Using a separate 27g. needle with a shield that limits the length of the needle exposed to 0.5mm, the skull was breached at the appropriate coordinates. The injection needle was advanced through the hole in the skull to a depth of 2mm from the skull surface and then backed-out 0.5mm to create a potential space. $1x10^5$ cells in a total volume of 2μL were injected over 1 minute. Following the injection, the needle was left in place for 3 minutes, and then slowly withdrawn. The skin was sutured closed.

Virus:

To produce virus stocks, sub confluent monolayers of baby hamster kidney 21 clone 13 (BHK) cells were infected with HSV strains in1314, 1716, dlspTK, or wild type 17+. Virus was concentrated from the culture and titrated by plaque assay as previously described (28). All viral stocks were stored frozen in viral culture medium (AUTO-POW media containing penicillin, and streptomycin) at -70°C, and thawed rapidly just prior to use.

Viral Inoculation:

Mice were anesthetized with I.M. ketamine/xylazine, and the head was cleansed with 70% EtOH. Using a Hamilton syringe with a 30g. disposable needle, the appropriate amount of virus was injected (10^4-10^6 PFU in 2μL) through a midline incision at the same stereotactic coordinates used for tumor cell injection. The injection was performed over 1 minute, and following the injection the needle was left in place for 3 minutes, and then slowly withdrawn.

Magnetic Resonance Imaging:

Mice were imaged using a 1.9 Tesla 30 cm bore animal MRI system located in the Hospital of the University of Pennsylvania MRI facility. Animals were anesthetized with I.M. ketamine/xylazine (87 mg/kg ketamine/13 mg/kg xylazine). Subsequently, each animal was injected with 10 units of Gd(DTPA) via a tail vein. The animal was taped in place within a plexiglass gradient coil and imaged.

Immunohistochemistry:

HSV-infected cells were detected by an indirect avidin-biotin immunoperoxidase method (Vectastain ABC Kit, Vector Labs, Burlingam, CA) as specified by the manufacturer with slight modification. Briefly, tissue sections were deparaffinized, rehydrated, quenched in peroxide (H_2O_2) and blocked in 3.5% goat

serum (Sigma Chem. Co., St. Louis, MO.). Sections were reacted overnight at 4°C with the primary antibody, a rabbit antiserum to HSV-1 (Dako Corp., Carpinteria, CA), used at a dilution of 1:1000. Next, the tissue was reacted at room temperature with biotinylated goat anti-rabbit IgG, the avidin-biotin horseradish peroxidase complex and finally AEC substrate. Sections were counter stained with hematoxylin and examined under the light microscope. As a control for the specificity of immuno-staining, tissues were processed as above, except that non-immune rabbit serum was substituted for the primary HSV-1 antiserum.

Titration of virus from tumor and brain:

Mice were sacrificed by lethal injection of anesthesia. Brains with or without in situ tumors were removed aseptically, snap frozen in liquid nitrogen, and stored at -70°C. Each tissue sample was rapidly thawed in a 37°C water bath, and the tissue was homogenized in viral culture medium at a 10% weight/volume ratio using a Pyrex Ten Broeck tissue grinder. The homogenates were centrifuged at 3,000 x g for 10 minutes at 4°C. The supernatant of each tissue homogenate was diluted logarithmically in media, and the viral titer of each was determined by plaque assay on BHK cells (28).

Statistics:

Standard deviation, and t-Test: two sample assuming unequal variances, were calculated using Microsoft Excel (Redmond, WA) on an apple MacIntosh computer (Cupertino, CA).

EXAMPLE 1 (lysis of melanoma cells)

In our initial studies, we wanted to make a straightforward in vitro determination of the relative abilities of HSV-1 wild type and mutant viruses to lyse various murine melanoma cells. We also wanted to compare how efficiently these melanoma cells were lysed by HSV-1 relative to baby hamster kidney (BHK) cells, which is a standard cell line used to propagate and titer HSV-1. Cells were plated in 24 well tissue culture plates at a density of $5x10^4$ cells/well. The viruses were diluted logarithmically and cell monolayers were infected in triplicate. After 72 hours of culture, the highest dilution of virus at which complete destruction of the monolayer still occurred, was recorded for each virus-cell combination. Data are expressed as the number of PFU of virus, obtained for each virus-cell combination.

As demonstrated in Table 1, the various mutant viruses lyse melanoma cells and BHK with efficiencies similar to wild type 17+. Cloudman S-91, and H-P melanoma cells were lysed efficiently relative to BHK.

EXAMPLE 2 (tumor production)

The capacity of each melanoma cell line to produce intracranial tumors was then evaluated. For each cell line, 10 C57B1/6 mice were injected stereotactically with $5x10^4$ cells in the right cerebral hemisphere. Mice were observed daily, and sacrificed when they appeared moribund, or after 6 weeks if they remained asymptomatic. Each brain was fixed, sectioned, stained, and examined histologically for tumor. Both H-P and B-16 formed intracranial tumors in 10 of 10 C57B1/6 mice, while Cloudman S-91 only formed a tumor in 1 of 10 mice.

We decided to proceed with the H-P model, since these cells were both susceptible to lysis by the relevant HSV-1 mutants, and formed brain tumors efficiently.

Subsequent experiments verified that stereotactic injection of H-P cells into the brain of C57B1/6 mice establishes tumors in 100% of the animals. A technical advantage of this system is that the presence of a brain tumor can be verified by magnetic resonance imaging (MRI) prior to treatment, or simply by observation of a pigmented area on the skull overlying the tumor site, generally by 5 days post cell injection. The tumors progressed to a size that caused the mice to become moribund from neurologic symptoms in approximately two weeks.

EXAMPLE 3 (treatment of brain tumors with HSV-1 mutant 1716).

C57B1/6 mice were injected stereotactically in the right cerebral hemisphere with 5×10^4 Harding-Passey melanoma cells. After 10 days (Figure 1a) or 5 days (Figure 1b), 5×10^5 PFU of HSV 1716 was injected at the same stereotactic coordinates. The number of days elapsed between injection of tumor cells and time mice became moribund is shown on the X axis. Control mice were injected with an equal volume of viral culture medium at the appropriate time.

As shown in Figure 1a stereotactic injection of HSV-1 mutant 1716 into brain tumors 10 days after establishment, resulted in a statistically significant increase in the length of time elapsed until the mice become moribund ($P(T<=t)$ one-tail: 1.016×10^{-4}). However, no long term survivors were obtained. When viral therapy was performed 5 days after tumor establishment (Figure 1b), significant improvement in outcome was again seen in the treatment group ($P(T<=t)$ one-tail: 7.707×10^{-3}), and 2/10 treated mice were cured. One long term survivor was sacrificed after day 36 post viral infection. Microscopic examination of serial sections of the brain did not reveal any residual tumor (data not shown). The second animal is still alive and asymptomatic at greater than 150 days post treatment. Treated animals that became moribund, showed progression of their brain tumors upon

examination of tissue sections.

EXAMPLE 4 (1716 replication in tumor and non-tumor cells).

Immunohistochemistry shows that replication of 1716 is in fact restricted to tumor cells, and does not occur in surrounding brain. A significant number of melanoma cells within tumor were stained by polyclonal antiserum to HSV-1 on days 3 and 6 post infection. Moreover, in tumor bearing mice treated with 1716, no HSV-1 antigen staining was seen in brain tissue adjacent to tumor or in any other areas of brain in all sections examined. In addition, no histologic evidence of encephalitis was seen in any 1716 treated mice at any time. In contrast, tumor bearing mice infected with wild type 17+ virus, exhibited multiple focal areas of HSV-1 immunohistochemical staining both within tumor and in surrounding and distant brain as well. A significant encephalitis characterised by polymorphonuclear leukocytes, nuclear dust, and extravasation of red blood cells, is seen in areas of this and other sections examined. In control experiments, no immunohistochemical staining was seen with anti-HSV-1 in tumor or brain from mice who did not receive virus, or in virally infected brain tumor sections subjected to the full immunohistochemical protocol with normal rabbit serum substituted for the primary anti-HSV-1

antibody (data not shown).

EXAMPLE 5 (kinetics of replication in tumor and non-tumor cells).

Having shown striking restriction of 1716 replication to tumor by immunohistochemistry, we next attempted to quantify the kinetics and extent of replication of 1716 in tumor by titration of infectious virus, and compare this with titration data from non-tumor bearing mouse brain infected with 1716 or 17+.

To investigate the extent of 1716 replication in brain tumors, C57B1/6 mice were injected with Harding Passey melanoma cells right of midline. Seven days later each mouse was infected with 5×10^5 PFU of 1716 at the same stereotactic coordinates. At the times indicated, mice were sacrificed, the brains were frozen in liquid N_2 and stored at -70°C. Specimens were thawed rapidly, homogenized, and viral titration was performed in triplicate on BHK cells (closed squares). These data represent the mean of 4 mice at each time point.

To establish the growth characteristic of 1716 and wild type 17+ in brain without tumor, mice were injected intracranially with either 5×10^5 PFU 1716 (open squares) or 1×10^3 PFU of 17+ (closed triangles). Mice were sacrificed at the times shown and processed as described above. Each point is the

mean of 2 mice.

As shown in Figure 2, wild type 17+ virus replicated efficiently in non-tumor bearing mouse brain. In contrast, no replication of 1716 occurred in brain of non-tumor bearing mice. The titer of virus recovered decayed over time, and infectious 1716 could only be isolated for 3 days after inoculation. However, when 1716 was injected into brain tumors, significant replication occurred as evidenced by recovery of an amount of infectious 1716 on day 1 post inoculation that is substantially greater than the input amount. Under these conditions, infectious 1716 could be isolated from tumor bearing mice for 5 days post inoculation, but not on day 7. These results clearly demonstrate that HSV-1 mutant 1716 will freely replicate in tumor cells (leading to their destruction) but does not replicate in non-tumor cells (leaving them unharmed).

Table 1: Relative susceptibility of melanoma cells to lysis by HSV-1.

Virus	Cell Type		
	Cloudman S91	Harding-Passey	BHK
in1814	10^4	10^4	10^3
dlspTK	10^3	10^4	10^3
1716	10^4	10^4	10^3
17+ (wild type)	10^3	10^3	10^2

EXAMPLE 6 (construction of HSV-1 mutant 1764)

An HSV-1 mutant referred to as 1764 was constructed by including a secondary gene mutation into the 1716 mutant. The secondary mutation was the modification of the UL48 gene coding for Vmw65 transinducing protein by the introduction of a 12-base pair insertion therein. The production of HSV mutant in1814 containing the UL48 mutation is described in C.I. Ace et al. J. Virol. 63, 2260-2269 (1989). A cloned fragment spanning UL48 containing the mutation was recombined into 1716 by standard techniques. Single plaques were isolated, their DNA profile analysed by Southern blotting and an isolate with the correct profile selected.

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Fig 1a

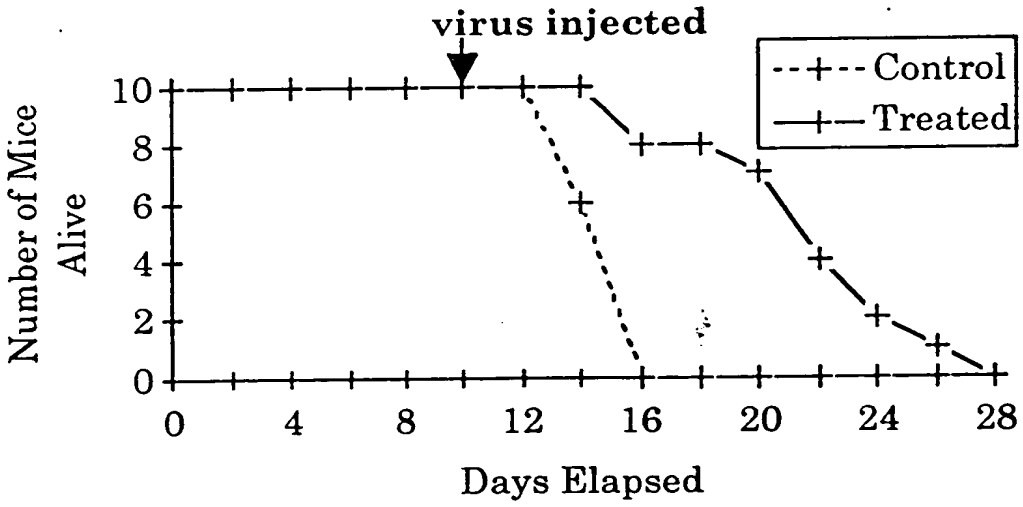

virus injected
Control
Treated
Number of Mice Alive
10
8
6
4
2
0
0
4
8
12
16
20
24
28
Days Elapsed

Fig 1b

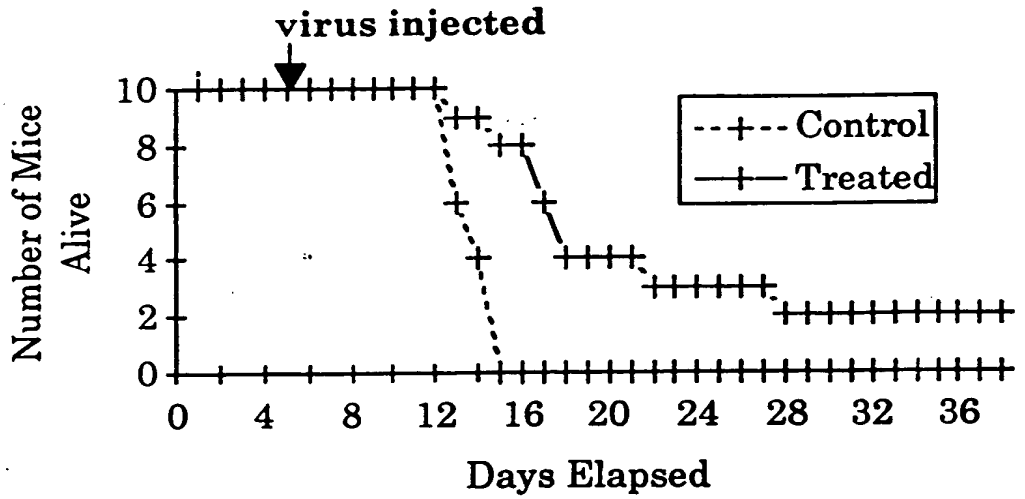
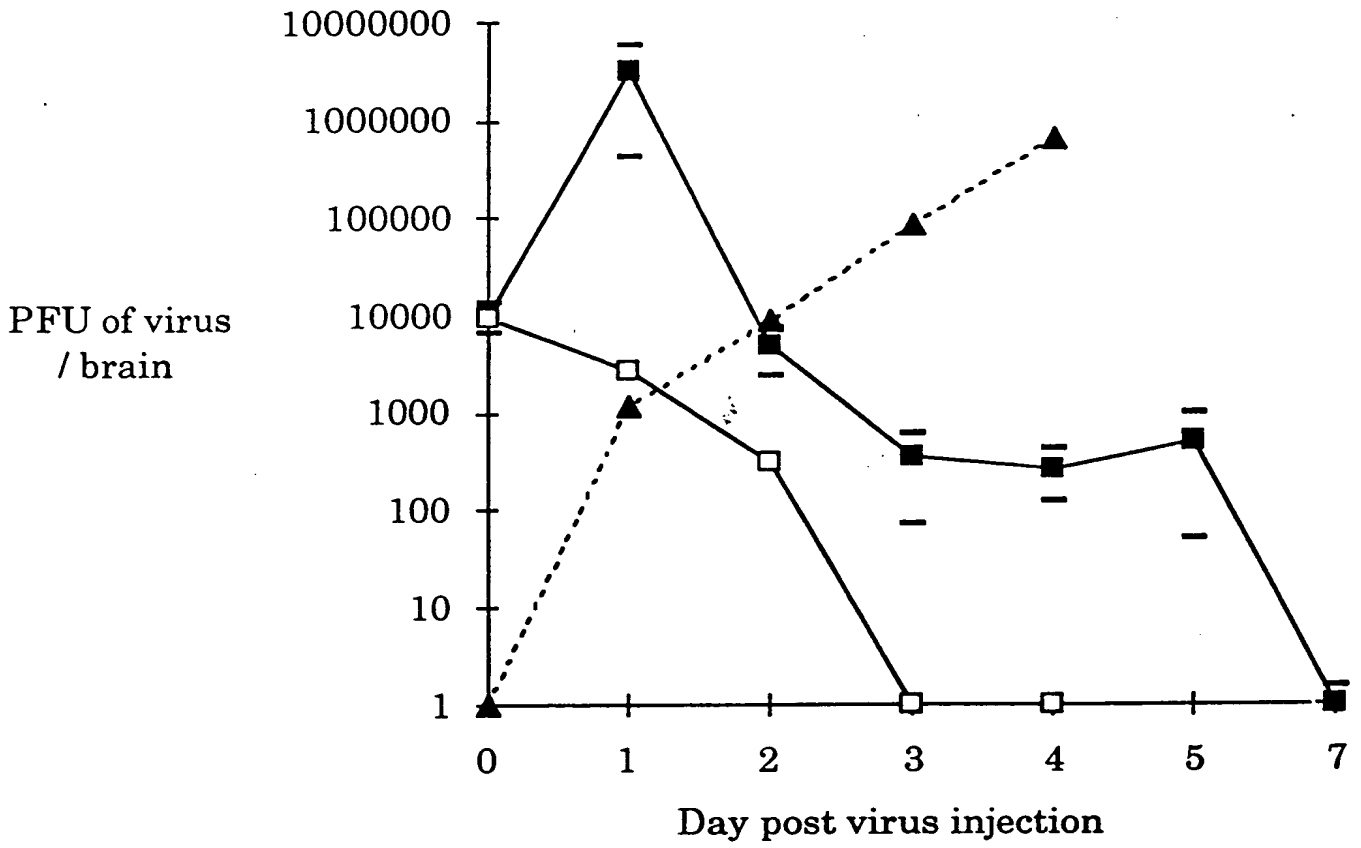

virus injected
Control
Treated
Number of Mice Alive
10
8
6
4
2
0
0
4
8
12
16
20
24
28
32
36
Days Elapsed

Fig 2


10000000
1000000
100000
10000
1000
100
10
1
PFU of virus / brain
0
1
2
3
4
5
7
Day post virus injection